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ROLE OF INTRACELLULAR pH ON GROWTH HORMONE RELEASING HORMONE (GHRH)-STIMULATED ADENOSINE 3'5'-MONOPHOSPHATE AND GROWTH HORMONE RELEASE FROM RAT ANTERIOR PITUITARY CELLS

BY

ANURADHA L. PUTTAGUNTA

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

FALL, 1991

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Abstract

In this study, the effect of changes in intracellular pH (pHi) on Growth Hormone Releasing Hormone (GHRH)-stimulated cAMP and GH release was examined using a static monolayer culture prepared from dispersed rat anterior pituitary cells. To modulate pHi, three approaches were used: inhibition of the Na^+/H^+ antiporter with 5-(N-methyl-N-isobutyl) amiloride (MIBA), dimethyl amiloride (DMA) and amiloride; variation of extracellular pH (pHo); and addition of sodium propionate and ammonium chloride which alter pHi directly. Direct pHi measurement with 2'7'-bis(carboxyethyl)-5(6)carboxyfluorescein showed that treatment with MIBA, DMA and amiloride reduced pHi by 0.05 units. Variation of pHo from 6.7 to 7.7 changed pHi from 6.85 to 7.35. Sodium propionate (30 mM) reduced pHi by 0.15 units and ammonium chloride (30 mM) increased pHi by 0.3 units. Studies on GHRH-stimulated cAMP release indicated that MIBA, DMA and amiloride inhibited GHRH-stimulated cAMP levels by up to 50%. Increased pHo from 6.6 to 7.8 enhanced GHRH-stimulated cAMP release by 80%. Reduction of pHi by sodium propionate dose-dependently inhibited GHRH-stimulated cAMP levels by up to 50% while ammonium chloride enhanced the GHRH-stimulated cAMP release by up to 75%. In the studies on basal and GHRH-stimulated GH release (15 min post GHRH stimulation), it was found that MIBA, DMA and amiloride inhibited GHRH-stimulated GH release by about 45%. Acute elevation of pHo from 6.6 to 7.8, in addition to increasing basal GH release by

6-fold, also enhanced the GHRH-stimulated GH release. Surprisingly, both ammonium chloride and sodium propionate dose-dependently inhibited GHRH-stimulated GH release. The effects of these modulatory approaches on GH release two hours after stimulation by GHRH revealed effects similar to the effects on GH release 15 minutes after stimulation. These results indicate that pHi acts as a modulator of GHRH-stimulated cAMP and GH release. However, since the modulating effects of pHi on GHRH-stimulated GH secretion do not always parallel changes in cAMP release, more than one intracellular signalling pathway involved in GH secretion is likely to be sensitive to changes in pHi.

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At the end, I would like to put in a word of appreciation for my husband for bearing with me.

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LIST OF ABBREVIATIONS

Amil:	Amiloride
BCECF:	2'7'-Bis(carboxyethyl)-5(6)-carboxyfluorescein
BSA:	Bovine Serum Albumin
Ca^{2+}:	calcium ion
$[Ca^{2+}]i$:	intracellular calcium
cAMP:	Adenosine 3'5' monophosphate
DMA:	Dimethyl amiloride
EGTA:	Ethyleneglycol-bis-(B-aminoethyl ether)-N,N'-tetraacetic acid
FCS:	Fetal Calf Serum
g:	gram
GH:	Growth Hormone
GHRH:	Growth Hormone Releasing Hormone
GppNHp:	5' guanylyl imido diphosphate
G_s:	Guanine nucleotide binding protein
h:	hour
H^+:	hydrogen ion
H^+_i:	hydrogen ion concentration inside the cell
H^+_o:	hydrogen ion concentration outside the cell
HCl:	hydrochloric acid
HEPES:	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonicacid
IBMX:	3-isobutyl-1-methylxanthine
K^+:	potassium ion
M:	Molar
µg:	microgram
µL:	microliter

μM:	micromolar
mCi:	Millicurie (37 megaBq)
mg:	milligram
min:	minute
MIBA:	5-(n-methyl-N-isobutyl) amiloride
mL:	milliliter
mM:	millimolar
NaCl:	sodium chloride
NaOH:	sodium hydroxide
Na^+:	sodium ion
Na^+_i:	sodium ion concentration inside the cell
Na^+_o:	sodium ion concentration outside the cell
NaP:	sodium propionate
NH_4Cl:	ammonium chloride
nm:	nanometer
nM:	nanomolar
O_2:	oxygen
PBS:	phosphate-buffered saline
PGE_2:	prostaglandin E_2
pH:	negative logarithm of hydrogen ion concentration
pHi:	Intracellular pH
pHo:	Extracellular pH
PKC:	protein kinase C
pM:	picomolar
PMA:	phorbol, 12-myristate, 13-acetate
RIA:	Radioimmunoassay
s:	second

SEM: standard error of the mean

SRIF: Somatostatin

I. INTRODUCTION

It has been generally accepted that intracellular pH (pHi) is an important regulator of many physiological functions (Madshus, 1988). Virtually all cellular processes have demonstrated pH-sensitivity. For instance, a small alkaline shift may accelerate such diverse processes as glycolysis, protein synthesis and cytoskeletal reorganization (Condeelis *et al.*, 1982; Fidelman *et al.*, 1982).

Recently, it has been demonstrated that pHi is involved in the regulation of the cyclic nucleotide second messenger system in neuroendocrine tissues (Ho *et al.*, 1989; 1990). The modulatory effect of pHi on hormone (glucagon) secretion has also been recently demonstrated by Morand *et al.* (1988). These findings suggest that the physiological function of endocrine organs may be pH-sensitive.

The physiological importance of pHi in the functioning of endocrine organs is also suggested by the observations that states of chronic acidosis and alkalosis in children are associated with attenuated growth (Rosenfield, 1988). With these observations in mind, it was hypothesized that one or more intracellular signalling pathways that regulate growth hormone (GH) secretion are likely to be sensitive to changes in pHi. These effects of pHi may, in turn, affect GH secretion.

The aim of this study was to determine the effects of alteration of pHi on: a) basal and Growth Hormone Releasing Hormone (GHRH)-stimulated cAMP release; and b) on basal and GHRH-stimulated GH release from rat anterior pituitary cell cultures.

I.1 Intracellular pH (pHi)

I.1.1 Physiological importance of pHi

In the human body, changes in pHi may have varied effects on endocrine functions. For example, it has been observed that most generalised metabolic disturbances attenuate growth. Alkalosis arrests growth unless well controlled with potassium chloride. Acidosis, such as that resulting from renal failure or renal tubular disease, causes growth retardation (Rosenfield, 1988).

Intracellular pH is important for the activity of a number of enzymes with pH optima within the physiological range (Fidelman *et al.*, 1982) as well as for the efficiency of contractile elements (Condeelis and Vahey, 1982) and the conductivity of ion channels (Moody, 1984). Also, pH oscillations seem to be important in controlling the cell cycle and the proliferative capacity of cells (Gillies and Deamer, 1979).

Because of the great importance of pHi for many cellular

processes, cytoplasmic pH is strictly regulated. Eukaryotic cells clamp cytoplasmic pH at 7.0 to 7.4 by ion transport mechanisms and a high buffering capacity of the cytoplasm (Aickin and Thomas, 1977).

I.1.2 Regulation of pHi

In the last few years, pHi regulation has been studied with great interest in a rapidly increasing number of cell types and tissues. Most eukaryotic cells appear to have two or more pH-regulating mechanisms and the interaction between the different mechanisms is complicated (Busa, 1986). The principal ion exchange mechanisms proposed to be involved in regulation of pHi include:

1) amiloride-sensitive Na^+/H^+ exchange;
2) Na^+/Cl^- co-transport functionally coupled to Cl^-/HCO_3^- exchange;
3) $Na^+/K^+/2Cl^-$ co-transport functionally coupled to Cl^-/HCO_3^- exchange;
4) Na^+/H^+, HCO_3^-/Cl^- double exchange; and
5) $NaCO_3^-$ pair formation and $NaCO_3^-/Cl^-$ exchange.

Among the H^+ extrusion systems proposed, the amiloride-sensitive Na^+/H^+ exchange system seems to represent the principal cellular pH-regulating system in vertebrate cells (Hofmann and Simonsen, 1989). This is suggested by the fact that the most important factor controlling the Na^+/H^+ exchange

under physiological conditions is pHi. Apart from the regulation of pHi and Na^+ concentration, the Na^+/H^+ antiporter has been implicated in a wide variety of functions, including the control of cell volume (Grinstein *et al.*, 1985a) and the initiation of growth and proliferation (Rothenberg *et al.*, 1983).

I.1.3 Principal features of the Na^+/H^+ antiporter

The first demonstrations of Na^+/H^+ exchange in eukaryotic plasma membranes were made in vesicles from the brush borders of rabbit kidney and small intestine (Hopfer and Kinne, 1976). Recently, much information has accumulated regarding the Na^+/H^+ exchanger in mammalian cells, and there seems to be a general agreement that all animal cells possess an electroneutral Na^+/H^+ antiporter (Krulwich, 1983). The antiporter responds to a fall in intracellular pH by quickly extruding protons in exchange with extracellular Na^+. The energy for the extrusion is provided by the large inward-directed Na^+ gradient.

In mammalian species, the stoichiometry of the Na^+/H^+ exchange process is one-for-one (Cala, 1980; Boron *et al.*, 1983). Consistent with this transport ratio, the antiporter is electrically neutral (Aickin *et al.*, 1977; Grinstein *et al.*, 1984a) and insensitive to maneuvers that alter the transmembrane potential (Kinsella *et al.*, 1981). However, in crustaceans, a transport stoichiometry of 2 Na^+/1 H^+ has been

demonstrated (Ahearn *et al.*, 1990). Since under physiological circumstances, sodium ion concentration outside ($[Na^+]_o$) >> sodium ion concentration inside the cell ($[Na^+]_i$) and hydrogen ion concentration outside ($[H^+]_o$) < hydrogen ion concentration inside the cell ($[H^+]_i$), the antiporter will normally operate in the Na^+_o/H^+_i exchanging mode (Aronson, 1985). However, under certain conditions, the direction of exchange can be reversed by inverting the direction of the gradient *i.e.* by removal of Na^+_o (Moolenar *et al.*, 1983).

The antiporter appears to be nearly quiescent when the pHi is in the physiological range. However, it can be activated by a wide variety of stimuli including hormones, growth factors (Moolenar *et al.*, 1986), tumor promoters (Grinstein *et al.*, 1985b; Besterman *et al.*, 1985) and hypertonic shrinking (Cala, 1980; Parker, 1983). The activation of the antiporter can lead to three immediate consequences: an alkalinization of pHi, an increased Na^+_i concentration and the uptake of osmotically obliged water (movement of water from an area of low osmolarity to an area of high osmolarity).

I.1.4 Experimental modulation of intracellular pH (pHi)

A number of mechanisms have been characterized to study pHi regulation. The cytosol may be acidified or alkalinized and studied under different conditions by a continuous

recording of pH (Grinstein and Dixon, 1989).

The Na^+/H^+ antiporter can be reversibly blocked by the diuretic, amiloride, or by more potent derivatives of amiloride, notably 5-(N,N-hexamethylene) amiloride and 5-(N-methyl- N-isobutyl) amiloride (Benos, 1982) (Appendix 1). Amiloride and its derivatives seem to compete with external Na^+ for binding to the external transport site. As a result, extrusion of protons by the antiporter is inhibited leading to acidification of the cytosol.

Another mechanism by which pHi can be regulated is to alter the extracellular H^+ concentration (pHo). Elevating the H^+_o concentration inhibits the forward Na^+_o/H^+_i exchange leading to cytoplasmic acidification (Aronson *et al.*, 1983). Competition with Na^+ for the transport site on the antiporter is probably the predominant factor in the inhibition (Kinsella *et al.*, 1981). Similarly, lowering the H^+_o concentration activates the Na^+/H^+ exchange promoting cytosolic alkalinization.

Cells can also be acid-loaded by exposure to a weak organic acid (sodium propionate), a technique described by Grinstein *et al.* (1984b). The cell penetration of the lipid-soluble undissociated free acid is followed by intracellular dissociation and cytoplasmic acidification, which, in turn, activates the exchange of Na^+_o for H^+_i.

There is now good evidence that the permeability of the cell membrane to at least one charged species, NH_4^+, is

sufficiently great for the passive movement of the ion to have significant second-order effects on pHi (Thomas, 1984). During a brief exposure to NH_3 and NH_4^+, the NH_3 rapidly enters and combines with H^+ to form NH_4^+, thereby raising the pHi. This alkalinization, however, is blunted as NH_4^+ also enters, though more slowly, and dissociates to form NH_3 and H^+.

I.2 Growth Hormone Secretion: Pathways involved

As its name implies, the primary function of GH is promotion of linear growth. Growth Hormone, via the somatomedins, increases protein synthesis by enhancing amino acid uptake and directly accelerating the transcription and translation of mRNA (Rosenfield, 1988). In addition, GH tends to decrease protein catabolism by mobilising fat as a more efficient fuel source. Reduced GH secretion in the body leads to dwarfism whereas excess GH secretion leads to acromegaly (Rosenfield, 1988).

The secretion of GH from the somatotrophs of the pituitary gland is controlled primarily by two hypothalamic peptide hormones, GHRH and somatostatin (SRIF); the former stimulates and the latter inhibits GH release (Vale *et al.*, 1983). Glucocorticoids and tri-iodothyronine also participate in the regulation of GH secretion by modulating the sensitivity and maximal response of somatotrophs to GHRH and possibly SRIF as well (Vale *et al.*, 1983).

Somatotrophs account for about 50% of the cell population in the pituitary gland (Kovacs, 1984). The GH present in the pituitary exists in two pools, a readily releasable pool and a storage pool (Stachura, 1982). Most of the hormone released into the blood by stimuli through the increase in GHRH levels appears to be from the releasable pool (Stachura, 1982).

The mechanisms whereby GHRH and SRIF exert their effects on GH secretion appear to involve multiple second messenger pathways. These include the adenylate cyclase - cAMP system (Sheppard *et al.*, 1979; Brazeau *et al.*, 1982; Kraicer and Chow, 1982); Ca^{2+} mobilisation (Schettini *et al.*, 1984; Lussier *et al., 1988*); increased membrane Na^{+} conductance (Kato *et al.*, 1989a; b); phospholipid hydrolysis (Canonico *et al.*, 1983); activation of protein kinase C (Limor *et al.*, 1989; Cronin *et al.*, 1985; 1986); and activation of the arachidonic acid cascade (Fafeur *et al.*, 1985; Bergeron and Barden, 1975).

I.2.1 Hormone/Receptor - G-protein - adenylate cyclase- cyclic AMP pathway

It is believed that GHRH first binds to specific sites on the plasma membranes of somatotrophs (Seifert *et al.*, 1985a; b), resulting in the stimulation of adenylate cyclase activity and consequent generation of cAMP. The elevated cAMP would then result (perhaps through the activation of a cAMP-dependent protein kinase or through elevation of $[Ca^{2+}]i$) in

growth hormone release via the process of exocytosis.

I.2.1.1 Role of Membrane receptor and Guanine Nucleotide Binding Protein G_s

The development of a GHRH radioligand binding assay demonstrated the existence of high affinity binding sites for GHRH in rat pituitary cells and homogenates (Seifert *et al.*, 1985a; 1985b). The specific binding of a radioligand to rat pituitary homogenates was found to be reduced by the addition of guanosine 5'-triphosphate (GTP) and its nonhydrolysable analogs 5' guanylyl-imido-diphosphate (GppNHp) and guanosine 5'-O-(3-thiotriphosphate). The effect of 0.1 μM of GppNHp on GHRH competitive displacements indicated a significant reduction in affinity for the ligand without an effect on the receptor number. The GHRH radioligand dissociates slowly from its receptor but the addition of 0.1 μM of GppNHp converts approximately half of the receptors present to a more rapidly dissociating form. These results are consistent with existing models for receptor/G-protein interactions, and thus, it was concluded that transduction of the GHRH receptors across the cell membrane involves a guanine-nucleotide protein, presumably G_s (Struthers *et al.*, 1989).

I.2.1.2 Role of Adenylate Cyclase

Several groups have reported that GHRH increases adenylate cyclase activity in a concentration-dependent manner in membrane preparations or homogenates obtained from rat anterior pituitary glands (Labrie *et al.*, 1983; Schettini *et al.*, 1984). These investigators made the following observations: a) GHRH causes guanine nucleotide-dependent, concentration-related stimulation of adenylate cyclase activity (Ka = 10^{-8} M); and b) guanine nucleotides were effective in stimulating cyclase in the absence of GHRH; the concentration of guanine nucleotides required for half maximal stimulation was decreased more than ten-fold in the presence of GHRH.

These observations suggested that coupling of the GHRH receptor to the adenylate cyclase catalytic unit occurs via a stimulatory guanine nucleotide regulatory protein, G_s. The results of parallel experiments demonstrated that in isolated somatotrophs, GHRH stimulation of GH release is preceded by a substantial elevation of cAMP concentration (Bilezikjian and Vale, 1983). Both adenylate cyclase activation and stimulation of GH release by GHRH are concentration-dependent.

I.2.1.3 Role of cAMP

Studies dealing with the regulation of GH secretion have suggested a role for cAMP as an intracellular mediator. Proof for the second messenger role of cAMP in the GHRH-stimulated GH release comes from the following studies: GHRH induces a concentration-related increase in adenylate cyclase activity in homogenates or particulate fractions obtained from whole pituitaries or tumour cell lines (Labrie *et al.*, 1983). GHRH increases GH release in a concentration-dependent manner, and the increase in GH release is preceded by an increase in cAMP accumulation (Sheppard *et al.*, 1985). Cyclic AMP analogs also increase GH release in a concentration-dependent manner (Sheppard *et al.*, 1980). The phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), increases GH release and potentiates the action of GHRH in increasing cAMP levels in the somatotrophs, with subsequent GH release (Bilezikjian and Vale, 1983). These observations support a second messenger role for cAMP in GHRH-mediated GH release.

I.2.2 Role of intracellular Ca^{2+} ($[Ca^{2+}]i$)

It was observed that Ca^{2+} is required in the incubation medium to elicit GH release from anterior pituitary cells (Sheppard *et al.*, 1980). Incubation of purified preparation of

somatotrophs in low Ca^{2+} medium (<85 μM) abolished the release of GH induced by the secretagogues PGE_2 and IBMX, while the increase in the intracellular cAMP was actually augmented (Spence *et al.*, 1980). It was also observed that verapamil (a Ca^{2+} channel blocker) inhibits GH release but slightly augments intracellular cAMP levels (Lussier *et al.*, 1988). These results suggested that Ca^{2+} is required to express the action of cAMP *i.e.* for the release process.

Using a combination of reverse hemolytic plaque assay and fluorescence microscopy with a Ca^{2+} indicator fura-2, it has been recently demonstrated that GHRH rapidly increases $[Ca^{2+}]i$, an effect which can be blocked by cobalt chloride, verapamil, or SRIF (Holl *et al.*, 1988). Forskolin and dibutyryl cAMP, two agents that elevate cAMP, also increase $[Ca^{2+}]i$ (Holl *et al.*, 1989). These findings suggest that the GHRH-induced $[Ca^{2+}]i$ rise is secondary to the influx of Ca^{2+} through membrane ion channels, and that cAMP may increase the influx of Ca^{2+} into the cytoplasm and thereby stimulate GH release.

I.2.3 Role of sodium ion (Na^+)

Recently, Na^+ has also been demonstrated by Kato *et al.* (1989a) to play an important role in GH secretion. They demonstrated that extracellular Na^+ is essential for GHRH and dibutyryl cAMP-induced GH secretion. By measuring changes in both the membrane potentials and $[Ca^{2+}]i$ with fluorescent dyes,

it was also demonstrated that a) GHRH depolarises rat somatotrophs and elevates $[Ca^{2+}]i$ and, b) this depolarisation and $[Ca^{2+}]i$ elevation is greatly suppressed by replacing extracellular Na^+ with mannitol or Tris (Kato *et al.*, 1989a). It was also observed that replacement of Na^+ with Li^+ (an alkali metal ion permeant to Na^+ channel) did not suppress GH secretion induced by either GHRH or dibutyryl cAMP (Kato *et al.*, 1989b) suggesting that depolarisation of the membrane is required for GH secretion.

Based on the above observations, it was proposed that GHRH depolarises somatotrophs by increasing membrane Na^+ conductance via cAMP, thereby activating voltage-sensitive Ca^{2+} channels, which, in turn, facilitate GH secretion (Kato *et al.*, 1989a; 1989b).

I.2.4 Role of Phosphatidylinositol Pathway

The involvement of the phosphatidylinositol pathway in the secretion of GH has been a controversial issue. One study demonstrated that natural and synthetic GHRH significantly stimulated phosphatidylinositol labelling at all times studied in a dose-dependent manner, suggesting that the phosphoinositol turnover may be involved in GH secretion in rat anterior pituitary cells (Canonico *et al.*, 1983). However, Escobar *et al.* (1986) demonstrated that GHRH has no effect on phospholipid labelling.

I.2.5 Role of Protein Kinase C (PKC)

It was observed that activators of PKC such as teleocidin and 4ß-phorbol 12-myristate 13-acetate (PMA) doubled the cAMP accumulation induced by GHRH, with no apparent effect on GHRH potency (Negro Vilar and Lapetina, 1985). An inactive 4-α-PMA had no such action in cultured anterior pituitary cells. This PMA potentiation could be measured as early as 60 sec, was maximal by 15 min, and waned by 3 to 4 h (Cronin *et al.*, 1986). A synthetic diacylglycerol, 1-oleoyl-2-acetyl glycerol (OAG) mimicked the action of tumor promoters. Due to the known stimulation of PKC by both tumor promoters and diacylglycerol it has been suggested that the Ca^{2+} and phospholipid-dependent PKC may enhance the ability of the GHRH receptor to activate the cAMP generating system (Cronin *et al.*, 1985).

Studies on GH release in primary rat pituitary cell cultures showed that PMA stimulated GH release 3- to 4-fold above the control value in a dose-dependent manner (Ohmura *et al.*, 1985). The effect of PMA on GH release could be detected as early as 15 min after the addition of PMA. These results suggest that one of the mechanisms of action of PMA can be attributed to the release of GH present in a storage pool. Furthermore, 8-Br-cAMP could not enhance maximally stimulated GH release by GHRH (Brazeau *et al.*, 1982) indicating that GHRH-stimulated GH release was through the cAMP pathway. However, dibutyryl cAMP could further stimulate the release of

GH beyond that observed using PMA alone. Similar additive effects were seen when GHRH was added with PMA. These observations are consistent with the possibility that PMA stimulates GH release via a different pathway from that of GHRH.

I.2.6 Role of Arachidonic Acid pathway

Prostaglandin E_2 (PGE_2) has been shown to be a potent stimulator of GH release (Shofield, 1970). Both GHRH and PGE_2 could stimulate cellular cAMP accumulation through GTP-dependent mechanisms (Labrie *et al.*, 1983). It has also been shown that both PGE_2 and GH were increased by GHRH in a concentration-related manner (Fafeur *et al.*, 1985). A significant correlation was observed between GH and PGE_2 release over the range of GHRH concentrations tested. Among the five prostanoids analyzed, only PGE_2 was selectively increased. Indomethacin and aspirin significantly reduced PGE_2 synthesis and GHRH-induced GH release. The inhibitory effect of indomethacin was counteracted by the addition of PGE_2 to the medium. This suggested that the effect of GHRH on GH release was at least partially under PGE_2 control. GHRH and PGE_2 at maximal concentrations had a partial additive effect on GH release, indicating that PGE_2 may facilitate GH secretion by increasing the sensitivity of the somatotrophs to GHRH.

Arachidonate and several of its prostaglandin metabolites have been well documented to markedly stimulate anterior pituitary adenylate cyclase activity (Mcleod *et al.*, 1970), cellular cAMP accumulation (Lefeure *et al.*, 1983) and GH release (Schofield, 1970). It has also been demonstrated that GHRH could increase arachidonate release from cultured anterior pituitary cells labelled with [^{3}H]-arachidonate in a concentration-dependent manner. Although the GHRH-induced increase in arachidonate release may affect GH release directly, it appears more likely that the stimulatory effect of GHRH is due to the metabolism of arachidonate to one or more subsequent metabolites (Judd *et al.*, 1985).

I.2.7 Regulation of GH Secretion by Somatostatin

Somatostatin is a tetradecapeptide first isolated from the hypothalamus on the basis of its ability to inhibit GH secretion by rat anterior pituitary cells in culture (Brazeau *et al.*, 1973). The mechanism whereby SRIF inhibits basal and GHRH-stimulated GH release has been the subject of many investigations. It has been demonstrated that although GHRH-stimulated GH release could be abolished by SRIF, cAMP accumulation, in either mixed pituitary cells or somatotrophs, was only slightly decreased (Bilezikjian and Vale, 1983; Michel *et al.*, 1983; Sheppard *et al.*, 1985; Narayanan *et al.*, 1989). ^{45}Ca-uptake studies have demonstrated that SRIF inhibits

basal and GHRH-induced increases in Ca^{2+}-influx and $[Ca^{2+}]i$ in somatotrophs (Lussier *et al.*, 1991a; b). Studies using the fluorescent dyes bisoxonol and quin 2 have shown that somatostatin increased K^+ conductance (Koch *et al.*, 1988). It has therefore been hypothesized that SRIF could prevent the GHRH-induced increase in $[Ca^{2+}]i$ by increasing K^+ conductance and, thus, hyperpolarising the somatotrophs. This inhibition of Ca^{2+} influx would thus inhibit GH release.

I.3 Proposed model for regulation of GH secretion

Based on the various studies done so far, the following model has been proposed for the regulation of GH secretion by GHRH and SRIF from somatotrophs (Lussier *et al.*, 1991c). GHRH, by binding to its receptor, stimulates the GTP-binding protein G_s (Narayanan *et al.*, 1989; Spiegel, 1987). This initiates one or more of the following interrelated events, leading to an increase in $[Ca^{2+}]i$ and, consequently, an increase in GH release. First, G_s interacts directly with L-type voltage-sensitive calcium channels (VSCC) via a membrane-confined mechanism to change their voltage sensitivity (Yatani *et al.*, 1988; Bean, 1989), thus causing the channels to open at resting membrane potential E_m, resulting in an increased Ca^{2+} influx. Second, G_s stimulates adenylate cyclase activity, resulting in an increase in cAMP accumulation (Sheppard *et al.*, 1985; Narayanan *et al.*, 1989). cAMP increases Na^+

conductance directly or via protein kinase A (PKA)-dependent phosphorylation (Nakamura *et al.*, 1987, Costa *et al.*, 1984). This increase in Na^+ conductance would lead to depolarisation and the opening of L-type voltage-sensitive calcium channels. The cAMP, via PKA-dependent phosphorylation, could allow L-type voltage-sensitive calcium channels to open when the cell is depolarised (Armstrong *et al.*, 1987). These events would result in a large and rapid increase in $[Ca^{2+}]i$, promoting GH release.

Somatostatin, on binding to its receptor, activates the pertussis toxin-sensitive GTP-binding protein G_i (Boyd *et al.*, 1988). G_i could interact directly with voltage-sensitive calcium channels through a membrane-mediated mechanism, which would result in a decrease in Ca^{2+} influx (Lewis *et al.*, 1986). The G-protein may be of the G_k type (Codina *et al.*, 1987), also pertussis toxin-sensitive, which would interact with K^+ channels to increase K^+ conductance. This would result in repolarisation or hyperpolarization of the cell, which would lead to the closing of the voltage-sensitive calcium channels. The resulting decrease in Ca^{2+} influx would lead to a decrease in $[Ca^{2+}]i$ and a subsequent reduction in GH release.

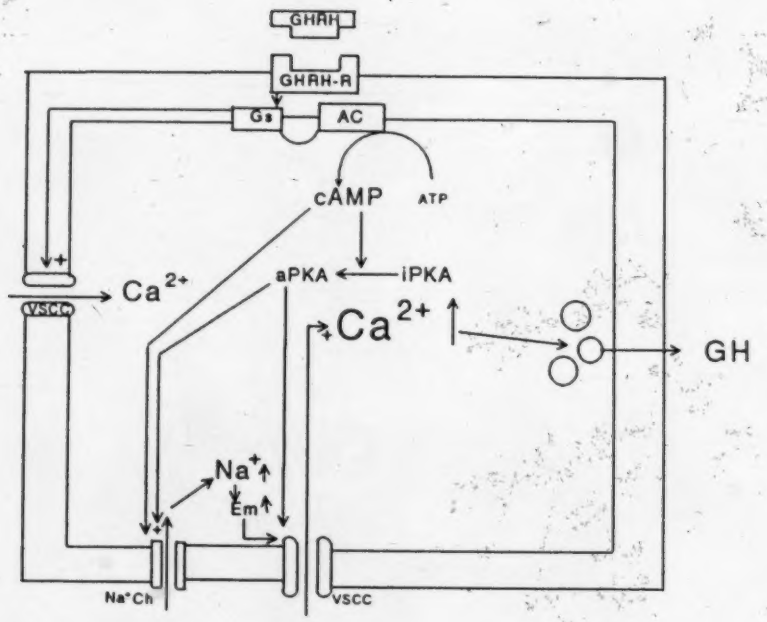

Figure 1. Model depicting the pathways responsible for the GHRH-stimulated GH release in rat somatotrophs. AC, adenylate cyclase; Em, membrane potential (↑, depolarisation); G_s, GTP-binding protein; GHRH-R, GHRH receptor; aPKA, active cAMP-dependent protein kinase; iPKA, inactive cAMP-dependent protein kinase; +, stimulation; Na^+Ch, sodium channel; VSCC, Voltage-sensitive calcium channel.

I.4 Relationship between pHi and pituitary function

In the human body, changes in pHi may occur secondary to a) fluctuation of extracellular pH as in states of acidosis and alkalosis; b) activation of the Na^+/H^+ antiporter; and c) ingestion of drugs such as salicylate (Moolenar *et al.*, 1983).

To our knowledge, the role of changes in pHi on the secretion of various pituitary hormones has not been investigated previously. The possible involvement of the Na^+/H^+ antiporter in the secretion of pituitary hormones, however, has been the subject of investigation in two recent studies. The first study demonstrated that in GH_4C_1 cells, activation of thyrotropin releasing hormone (TRH) receptors leads to a transient rise in $[Ca^{2+}]i$ which, in turn, leads to activation of the antiporter, and cellular alkalinization. The TRH-mediated effect is Na^+-dependent and can be inhibited by amiloride (Tornquist and Tashjian, 1991).

In the second study, the role of the Na^+/H^+ antiporter on luteinizing hormone (LH) release was evaluated using a series of amiloride derivatives. The amiloride derivative with potent inhibitory action on the Na^+/H^+ antiporter had a marked inhibitory effect on the GnRH-stimulated LH release (McArdle *et al.*, 1991). Therefore, it appears likely that the Na^+/H^+ antiporter may also be of importance in the regulation of GH secretion.

I.5 Relationship between pHi and second messengers

In the pineal gland, a neuroendocrine organ, it has been demonstrated that activation of the α_1-adrenergic receptor leads to cytoplasmic alkalinization via a PKC-dependent pathway. Cytoplasmic alkalinization is accompanied by enhanced norepinephrine-stimulated cAMP response while reducing pHi (using sodium propionate or via inhibition of the Na^+/H^+ antiporter by treatment with amiloride) leads to the reduction of the adrenergic-stimulated cyclic nucleotide responses (Ho *et al.*, 1989; 1990). These findings suggest that the receptor-mediated cyclic nucleotide responses are pH-sensitive and the Na^+/H^+ antiporter is an important regulator of these responses.

The relationship between cAMP and pHi varies depending upon the cell type. In lymphocytes and macrophages, a cAMP-sensitive mechanism has been shown to activate the Na^+/H^+ antiporter (Grinstein *et al.*, 1987; Kong *et al.*, 1989). In fibroblasts and vascular smooth muscle cells, a pertussis toxin-sensitive G-protein has been implicated in the activation of the Na^+/H^+ antiporter (Huang *et al.*, 1987; Paris *et al.*, 1983). On the other hand, in epithelial cells and cultured opossum kidney cells, cAMP has an inhibitory action on the Na^+/H^+ antiporter (Reuss and Peterson, 1985; Moran *et al.*, 1988).

The relationship between pHi and $[Ca^{2+}]i$ appears to be complex. In some tissues, changes in $[Ca^{2+}]i$ occur secondary

to changes in pHi (Grinstein and Goetz, 1985c; Moolenar *et al.*, 1984), whereas, in other tissues, agents that increase $[Ca^{2+}]i$ lead to activation of the Na^+/H^+ antiporter (Benos, 1982; Moolenar *et al.*, 1983; Owen and Villereal, 1982). Thus it seems that the temporal relationship between pHi and $[Ca^{2+}]i$ varies from tissue to tissue.

Several studies have indicated that PKC may be an important regulator of pHi. In smooth muscle cells, lymphocytes, fibroblasts and neuroblastoma cells, activation of PKC leads to cytoplasmic alkalinization (Grinstein *et al.*, 1985a; b; Moolenar *et al.*, 1984; Shimada and Hoshi, 1988; Too *et al.*, 1987). In vascular smooth muscle cells a PKC-independent mechanism is involved in the angiotensin or thrombin activation of the Na^+/H^+ antiporter (Berk *et al.*, 1987; Huang *et al.*, 1987). In human fibroblasts it has been suggested that activation of the Na^+/H^+ antiporter may involve the action of phospholipase A_2 (PLA_2) (Villereal and Owen, 1984).

Thus, the relationship between pHi and the various second messengers is complex and varies depending on the cell type.

I.6 Objective of Study and Experimental Strategy

In this study, an attempt has been made to determine how changes in pHi can modulate receptor-mediated intracellular mechanisms, particularly in the somatotrophs. Studies have

also been carried out to determine how pHi is regulated in somatotrophs and to elucidate the modulatory effects of pHi on GH secretion from the anterior pituitary cells.

To modulate pHi (pHi), different strategies were employed. One of the important regulators of pHi is the Na^+/H^+ antiporter. This antiporter appears to be present in most, if not all animal cells, where it has been implicated in a variety of functions, including the regulation of the cytoplasmic pH and Na^+ concentrations, the control of cell volume (Grinstein *et al.*, 1985a) and the initiation of growth and proliferation (Rothenberg *et al.*, 1983).

The Na^+/H^+ antiporter can be reversibly blocked by the diuretic amiloride or by more potent amiloride derivatives (Benos, 1982). So, as the first approach to modulate pHi, amiloride derivatives were used to inhibit the Na^+/H^+ antiporter. To determine the relationship between the effects of the amiloride derivatives and their potency in inhibiting the antiporter, three amiloride derivatives with varying potency in inhibiting the antiporter were used. In decreasing order of potency these were 5-(N-methyl-N-isobutyl) amiloride (MIBA), dimethyl amiloride (DMA) and amiloride (Appendix 1). The effects of these agents on pHi, cAMP and GH release from the anterior pituitary cells were determined. Apart from their inhibition of the Na^+/H^+ antiporter, amiloride and its derivatives have been demonstrated to have other effects (Anand-Srivastava, 1989; Frelin *et al.*, 1970). Therefore,

other methods were also chosen to confirm the findings.

Another method to vary the pHi is to vary the extracellular H^+ ion concentration. The second approach therefore was to vary the extracellular pH and study the consequent effects on basal and GHRH-stimulated cAMP and GH secretion.

The third approach was to directly alter the pHi using two modulating agents, sodium propionate and ammonium chloride. Using a fluorescent pH indicator, BCECF, a reduction in pHi was observed in the pituitary cells upon stimulation with sodium propionate and a rise in pHi was observed on stimulation with ammonium chloride. Therefore, the effects of these two modulating agents on cAMP and GH release were studied.

The hypothesis therefore was: pHi is an important regulator of GH secretion and one or more intracellular signalling pathways involved in GH secretion are likely to be sensitive to changes in pHi. To test this hypothesis, I chose the above three approaches to modulate pHi. The specific objectives of the study were:

a) to determine whether the three modulatory approaches chosen were indeed effective in altering pHi;
b) to determine the effects of these modulatory approaches on basal and GHRH-stimulated cAMP release; and
c) to determine the effects of these modulatory approaches on basal and GHRH-stimulated GH release.

II. MATERIALS AND METHODS

II.1 Cell Culture

a) Enzymatic dissociation by trypsinization

Male Sprague-Dawley rats (180-200 g) were decapitated, the pars nervosa-intermedia were discarded, and the anterior pituitary glands were collected in ice-cold phosphate-buffered saline (Appendix 2). The glands were washed 3 times with phosphate-buffered saline (PBS) and then minced into small fragments. The fragments were then transferred to digestion medium consisting of Dulbecco's Modified Eagle Medium (DMEM) with trypsin (1 mg/mL) and DNAse (0.01%, vol/vol). The glands were allowed to incubate in the medium at 37°C in a shaking water bath. After 20 min, the reaction was stopped with fetal calf serum (10%, vol/vol). The entire solution was then centrifuged at 700 x g for 7 min. The supernatant was discarded and the pellet was resuspended in PBS. Dispersion was carried out by triturating the glands with a pipette. The chunks were allowed to settle down and the supernatant was transferred to a centrifuge tube. The trituration was repeated until all the tissue was dispersed. The cell suspension was then centrifuged at 700 x g for 7 min. The supernatant was discarded and the pellet was resuspended in culture medium

(DMEM with Fetal Calf Serum [FCS]). The cells were washed twice and resuspended in the culture medium. Cell count and viability were determined using trypan blue dye exclusion method. Cell count was about 10^6/gland. Viability was about 90%. The cells were then plated onto multiwelled dishes at a density of 3 x 10^5 cells/well in 0.3 mL of culture medium. They were then incubated under a humidified atmosphere of 95% air/5% CO_2 at 37°C. After 48 h, the cells were washed three times with DMEM (without FCS) and equilibrated for 30 min before performing the experiments.

b) Enzymatic dissociation using papain

The anterior pituitary glands were collected in PBS as previously described. The glands were washed and minced into small fragments. The fragments were then transferred to a fresh enzyme solution consisting of Earl's balanced salt solution (EBSS) (Appendix 3) with 0.01% (vol/vol) DNAse and 20 U/mL papain. The glands were allowed to incubate in a shaking water bath at 37°C for 1 h. The tissue was then triturated and spun at 700 x g for 7 min. The pellet was resuspended in 3 mL of gradient solution "1/1" consisting of 1:10 dilution of gradient solution "10/10" [EBSS with albumin (10 mg/mL) and trypsin inhibitor (10 mg/mL)] with 0.01% (vol/vol) DNAse. The tissue was triturated again and the undissociated cells were allowed to settle to the bottom. The supernatant was overlayed

onto gradient solution "10/10". The above was spun at 200 x g for 10 min. The pellet was resuspended in culture medium and the cell count was done. The cell yield was 1.5 x 10^6/gland and the viability was 95%. Cells were plated as described above and experiments performed after 48 h.

This second method was chosen later for cell preparation because of the higher cell yield and better viability. The experiments performed with the cells prepared by trypsinisation were repeated to make sure that there was no changes due to change in cell preparation.

II.2 Materials

Synthetic rat GHRH (Peninsula Laboratories, Belmont, CA) was initially dissolved and stored in 0.1 M acetic acid at a concentration of 10^{-5} M. Further dilutions were made in sterile double-distilled water. Ammonium chloride and sodium propionate were obtained from Fluka Chemical Corp. (Ronkonkoma, NY). Trypsin, DNAse, papain, albumin, and trypsin inhibitor for the cell preparation were obtained from Sigma Chemical Corp. (St. Louis, MO). BCECF (2'7'-Bis(carboxyethyl)-5(6)-carboxyfluorescein), the acetoxy-methyl ester of BCECF (BCECF/AM), fura-2 and the acetomethyl ester of fura-2 (fura-2 AM) were purchased from Molecular Probes Inc. (Eugene, OR). RPMI-1640 medium without bicarbonate and Dulbecco's modified Eagle medium (DMEM) were purchased from Gibco (Grand Island,

NY). Nigericin, Triton X-100, EGTA, manganese chloride and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Corp. (St. Louis, MO). ^{125}I-cAMP was obtained from Bionetics Research Laboratories (Rockville, MD). ^{125}I-labelled NaI for iodination of GH was obtained from Amersham (Oakville, Ontario). Chloramine-T and sodium meta-bisulphite were obtained from Fisher Scientific Co. (NJ, NY). Amiloride derivatives were obtained from E. Cragoe Jr. (Nacogdoches, TX). The derivatives were initially dissolved in DMSO and the final concentration of DMSO in the incubation system was less than 0.1%. All the other drugs and chemicals were obtained from commercial sources and were of the purest grade available. Antibodies for the RIA of cAMP were gifts from Dr. A. Baukal (National Institutes of Health, Bethesda, MD). The antigen, antiserum and the reference preparation for the RIA of GH were obtained from National Institute for Arthritis, Diabetes, Digestive and Kidney Disease (NIADDK, Baltimore, Maryland). Sheep anti-monkey antiserum and normal monkey serum were kind gifts of Dr. G.M. Brown (Clark's Institute of Psychiatry, Toronto, ON).

II.3 Experimental Design

Studies with anterior pituitary cells have demonstrated that dibutyryl cAMP-mediated GH release was biphasic: an initial rapid rate of hormone release at 15 to 20 min which

was followed by a second release beginning at approximately 60 min (Stachura, 1976). Such phasic release was consistent with similar observations by other investigators with other secretagogues and other hormones held in granular storage (insulin, glucagon, vasopressin, and luteinizing hormone), and suggested the availability for release of two pools of stored hormone (Stachura, 1982). The first or labile pool was available for immediate release, while the second pool was available for prolonged release at an increased rate under constant stimulation.

With these findings in mind, it was decided to study the effects of modulation of pHi on GHRH-stimulated GH release at two time points: at 15 min for release of the labile pool and at 2 h for the study of release of the late pool.

The pituitary cells plated at a concentration of 3×10^5 cells/well were treated with GHRH at a varying concentration of 100 pM to 100 nM. At the end of 15 min, the medium was removed and assayed for cAMP release. The cells plated were lysed by alternate freezing and thawing and intracellular accumulation of cAMP was then measured. Figure 2 demonstrates that there is a parallel relationship between intracellular cAMP accumulation and cAMP released into the medium. Therefore, in experiments thereafter, cAMP released into the medium was measured as a reflection of the intracellular changes.

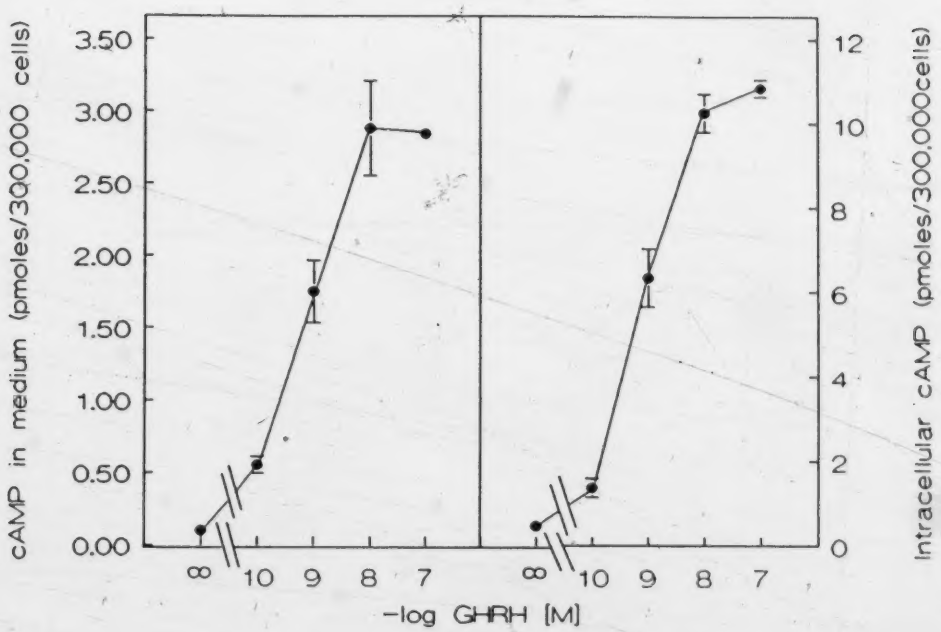

Figure 2. Relationship between cAMP released into the medium and intracellular cAMP accumulation. The cells were treated with GHRH at varying concentrations (100 pM-100 nM). After 15 min, the medium was removed and assayed for cAMP release. The cells were disrupted by alternate freezing and thawing and the intracellular cAMP accumulation was assayed.

II.4 Radioimmunoassay for cAMP

The medium bathing the cells was replaced by medium in which the drugs were dissolved to the required concentration. After 15 min, the medium was removed and assayed. For cAMP measurements, the medium was boiled for 5 min and assayed using a radioimmunoassay procedure in which samples are acetylated prior to analysis (Harper and Broker, 1975; Ho *et al.*, 1989). Intra- and interassay coefficients of variation were < 10%. All data are presented as mean $\pm$ standard error of the mean (SEM) of cAMP concentration levels in four aliquots of wells. Data were analyzed by Duncan's multiple range test (Duncan, 1955). The results are expressed as picomoles/300,000 cells.

II.4 Radioimmunoassay for Growth Hormone

Growth Hormone measurements were made at the end of 15 min as well as after a treatment period of 2 h. Growth Hormone was assayed using the National Institute for Arthritis, Diabetes, Digestive and Kidney disease rat radioimmunoassay kits. Growth Hormone was assayed in quadruplicate by the double antibody RIA and the results are expressed as micrograms/300,000 cells.

II.4.1 Iodination Procedure

Growth Hormone was iodinated with ^{125}I using the chloramine-T method (Hunter and Greenwood, 1962), and the labelled hormone was separated from the unlabelled hormone by gel filtration chromatography. The gel for the iodination column was prepared by dissolving 9.5 g of Sephadex G-75 in 300 mL of PBS and degassing it overnight at room temperature. The gel was then poured into the iodination column and allowed to pack. The top of the gel was then layered with 1 cm of sand (Fisher) and eluted with 1% BSA to reduce nonspecific binding. The rat GH (rGH) was solubilized in 0.01 M $NaHCO_3$ at 100 μg/mL and 50 μL of this solution was used for iodination. The radioactive iodide (0.5 mCi ^{125}I/10 μL of 0.5 M sodium phosphate buffer, pH 7.5) was added to the reaction chamber containing 50 μL of rGH solution and 50 μL of 0.5 M sodium phosphate buffer. The iodination reaction was initiated by adding 25 μL of freshly prepared chloramine-T (10 mg/10 mL). After exactly 25 s, the reaction was terminated by adding 25 μL of sodium metabisulphite (20 mg/10 mL) and 200 μL of 1% (wt/vol) BSA. The contents of the reaction mixture were then transferred to the column and eluted with the PBS-BSA solution. One hundred and sixty fractions were collected at 40 drops/tube for the first 10 tubes and 10 drops/tube for the next 150 tubes. Every fifth fraction was counted and the resultant data (counts per min) plotted on a graph. Binding

assays using radioimmunoassay were then performed on the peak fractions. The fractions with the best binding were pooled, aliquoted and stored at -4°C.

II.4.2 Radioimmunoassay procedure

For the RIA of GH, a 3-day double antibody radioimmunoassay protocol was used. On the first day, the standards and the samples were added to glass test tubes followed by the buffer (1% BSA in 0.025 M EDTA phosphate-buffered saline, pH 7.6, Appendix 4). The antibody (monkey anti-rGH) was then added and allowed to incubate at -4°C for 24 h. On the second day, the radiolabelled ligand was added to all the tubes which were then allowed to incubate at room temperature overnight. On the third day, the second antibody (sheep anti-monkey gamma globulin), the carrier (normal monkey serum) and polyethylene glycol were added. After incubation at room temperature for 2 h, the tubes were centrifuged at 3000 x g for 25 min at 4°C. The supernatant was discarded and the precipitate (pellet) was assessed using a gamma counter (Cobra Auto-gamma, Canberra Packard, Mississauga, ON).

II.5 Determination of intracellular pH

Intracellular pH was determined using a fluorescence pH indicator, BCECF. Briefly, 5 x 10^5 cells were pelletted, and

resuspended in culture medium (DMEM with HEPES, pH 7.2). The cells were loaded with BCECF by incubation with 2 μM BCECF/AM for 45 min at 37°C. The cells were then pelleted, washed twice and resuspended in HEPES-buffered RPMI-1640 (Appendix 5) (3×10^5 cells/mL). Aliquots of this suspension (1.5 mL) were transferred to a cuvette for the fluorometric determination of pHi, using a SLM Aminco DMX1000 fluorescence spectrophotometer with a thermostatically controlled cell holder fitted with a magnetic stirrer.

The pHi of pituitary cells was determined by monitoring the ratio of the fluorescence emission signal at 535 nm (8-nm slit width), with the excitation wavelengths set at 500 nm and 450 nm (8-nm slit width). The temperature was maintained at 37°C. The fluorescence signal was recorded continually with a computer. Calibration of pHi versus fluorescence signal was carried out at the end of each experiment by disrupting the cells with the detergent Triton X-100 (0.01%, vol/vol in water), followed by titration of the medium with aliqouts of diluted NaOH or HCl. Under this condition, the BCECF fluorescence varied linearly with pH in the range 6.4 to 7.7 and calibration was done by reading pH values from a linear plot of fluorescence intensity versus medium pH. Interference due to autofluorescence and light scatter of unloaded cells represented < 1% of the total signal of BCECF-loaded cells. It has been reported (Rink *et al.*, 1982) that the fluorescence emitted by intracellularly trapped fluorescein derivatives was

somewhat decreased compared with the free dye because of a red shift. Therefore, a correction must be introduced if calibration was made after cell lysis. The necessary correction factor was determined by the method of Thomas *et al.* (1979), using nigericin according to the equation $(H_i^+)/H_o^+ = K_i^+/K_o^+$ (H_i^+ and H_o^+ being the concentration of hydrogen ion inside and outside the cell and K_i^+ and K_o^+ being the potassium ion concentration inside and outside the cell). If the cells were suspended in K^+ solution, H_i^+ would follow H_o^+. In the presence of nigericin, disruption of the pituitary cells resulted in an increase in the fluorescence signal, which confirmed the occurrence of intracellular quenching. In the pituitary cells, the fractional fluorescence increase was constant in the pH range of 6.15 to 7.8 with a correction factor of 0.42 ± 0.012.

II.6 Determination of intracellular Ca^{2+}

The procedure for loading pituitary cells with fura-2 AM was similar to that for loading with BCECF/AM and the concentration of fura-2 AM used was 5 μM. After washing twice with DMEM medium, the pituitary cells were suspended in fura-2 medium (Appendix 6). After dye loading, 5×10^5 cells in 1.5 mL were used for fluorescence signal determination. The conditions and procedure were similar to those for the pHi measurement except that the excitation wavelengths were 380 nm

and 340 nm and emission was monitored at 510 nm. Free Ca^{2+} concentrations were calculated according to the equation established by Poenie *et al.* (1985): $[Ca^{2+}]i = K_d \times F_o/F_s \times (R - R_o) / (R_s - R)$ where K_d is the dissociation constant of fura-2-Ca^{2+} complex (225 nM), F_o and F_s are the fluorescence intensities at 380 nm for free (o) and Ca^{2+}-saturated (s) dye, R, R_o and R_s are the ratio of the dye fluorescence intensities at 340 nm and 385 nm for unknown, free and Ca^{2+}-saturated dye, respectively. Both F_s and R_s were determined by lysing the cells with Triton X-100 (0.1%) while F_o and R_o were determined by addition of 10 mM EGTA to the lysed cell suspension.

II.7 Statistical analysis:

Data are presented as the mean $\pm$ SEM of the amount of cAMP or GH in four aliquots of cells. The amount of cyclic nucleotide and GH in each well was based on duplicate determinations. Data were analyzed by Duncan's multiple range test (Duncan, 1955).

III. RESULTS

III.1. Effects of various modulating agents on pHi

In several cell types, hormones or growth factors induce a rapid rise in pHi (Tornquist and Tashjian, 1991; Moolenar, 1986). To determine whether GHRH had a similar effect on somatotrophs, the cell culture approach described in Materials and Methods was used. Anterior pituitary cells were loaded with the fluorescent indicator, BCECF/AM. Spectrophotometer studies with these cells were then performed to determine the pHi as described in the Materials and Methods section. At the pHo of 7.4, pHi stabilised at 7.1 ± 0.04. Addition of GHRH (1 nM-100 nM) did not alter basal pHi (Figure 3).

The next step was to determine whether the modulatory approaches chosen were effective in altering pHi.

First, the effects of three inhibitors of the Na^+/H^+ antiporter (amiloride and its two derivatives, MIBA and DMA) on pHi were determined. At pHo of 7.4, pHi stabilised at 7.1 ± 0.04. Treatment with amiloride, dimethyl amiloride (DMA) and 5-(N-methyl-N-isobutyl) amiloride (MIBA) reduced pHi by 0.05 ± 0.01 units (Figure 4), thus indicating the presence of an amiloride-sensitive Na^+/H^+ antiporter in the anterior pituitary cells.

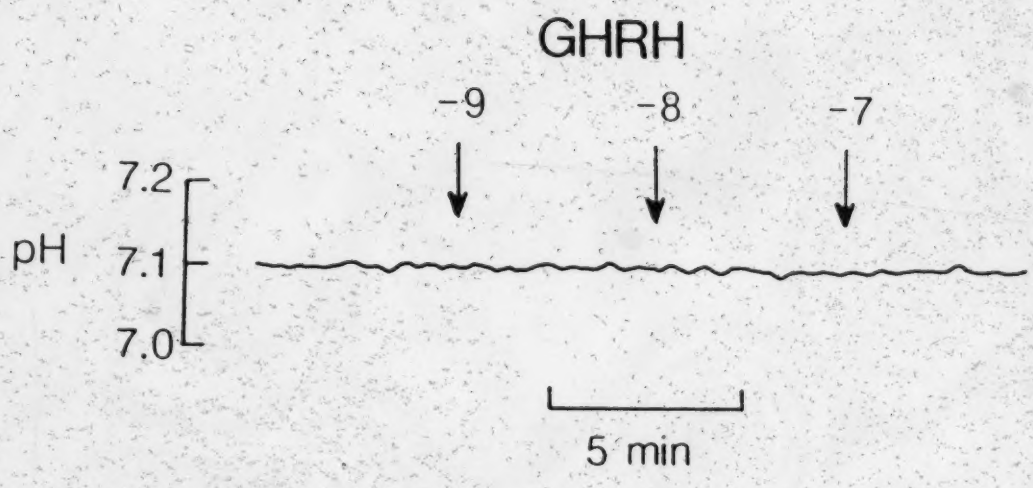

Figure 3. Effects of GHRH on pHi. The anterior pituitary cells were prepared and loaded with the fluorescent pH indicator BCECF as described in Materials and Methods. The ratio of the fluorescence emission signal at 535 nm, excited at 500 and 450 nm was continually recorded and calibrated as described. After the basal pH stabilised, GHRH (10^{-9} M - 10^{-7} M) was added. The trace is representative of six experiments.

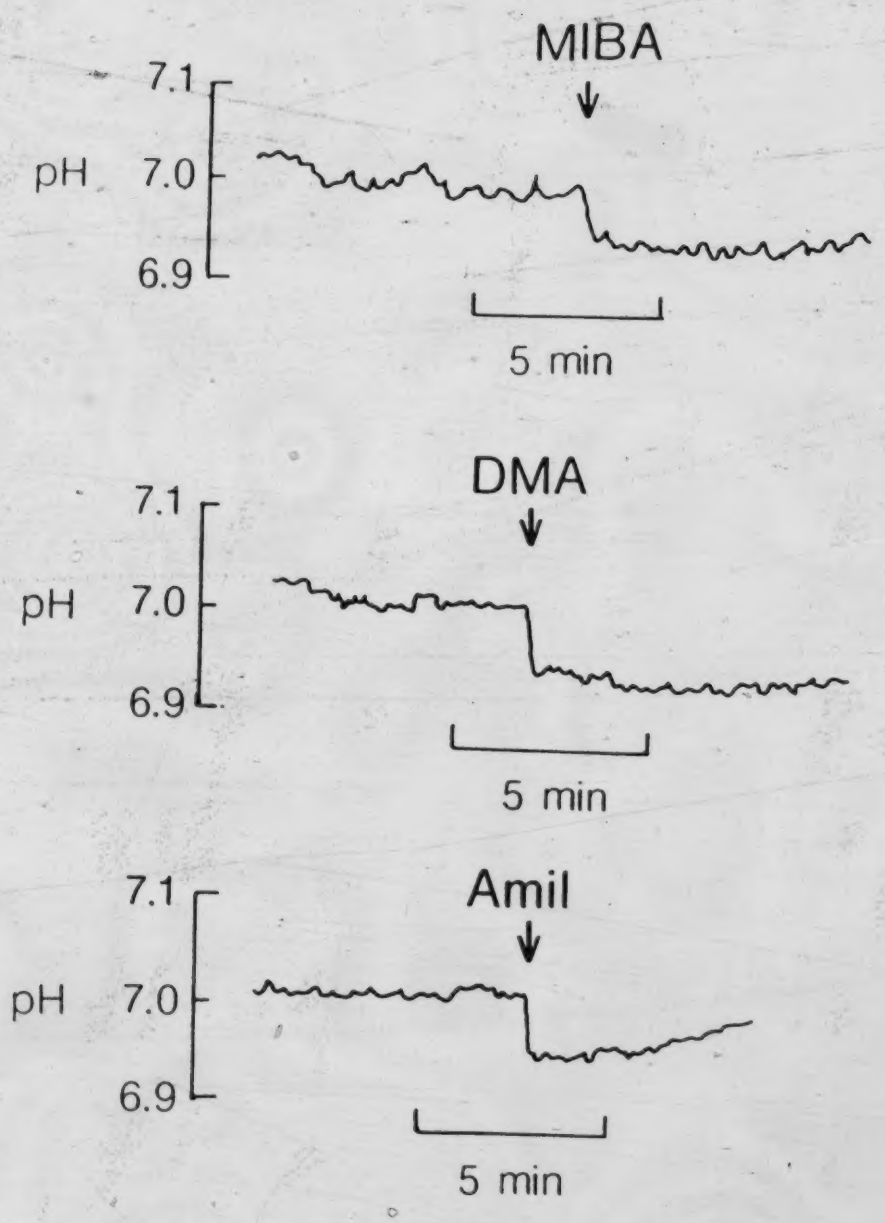

Figure 4. Effects of inhibition of the Na^+/H^+ antiporter on pHi. To determine the effects of inhibition of the Na^+/H^+ antiporter, amiloride (Amil) and its two derivatives MIBA and DMA were used. After the basal pHi stabilised, MIBA, DMA and amiloride at a concentration of 10 μM were added and the pHi recorded. The traces are representative of three experiments.

Second, to determine if pHi varied with variation of pHo, the relationship between extracellular and intracellular pH was determined. External pH was varied from 7.8 to 6.7 using 0.5 M HCl and the corresponding pHi was recorded. Figure 5 demonstrates the relationship between pHo and pHi after correction for the red shift (see Materials and Methods). For a pHo change of 1 unit, the pHi changed only by 0.48 units. The graph demonstrates that within the limits of 6.8 and 7.6 (pHo), the pHi varies linearly with pHo. However, beyond these limits, the pituitary cells maintain their physiological pH, presumably, through the Na^+/H^+ antiporter. This confirmed that pHi could be modulated by variation of pHo within the limits of 6.8 to 7.6.

The effect of ammonium chloride and sodium propionate on pHi was then determined in BCECF-loaded anterior pituitary cells. At a pHo of 7.4 the pHi stabilised at 7.1 $\pm$ 0.04. Treatment with ammonium chloride at a concentration of 30 mM produced an immediate rise in pHi to 7.4 $\pm$ 0.03, *i.e.* by 0.3 units. This rise in pHi was maintained for the duration of the tracing which was allowed to run for 15 min (Figure 6). In contrast, addition of sodium propionate at a concentration of 30 mM produced a sustained fall in pHi by 0.15 $\pm$ 0.02 units. To confirm that these changes in pHi were not due to the changes in intracellular osmolarity, sodium chloride at a concentration of 30 mM was added. There was no change in pHi on addition of sodium chloride (Figure 6).

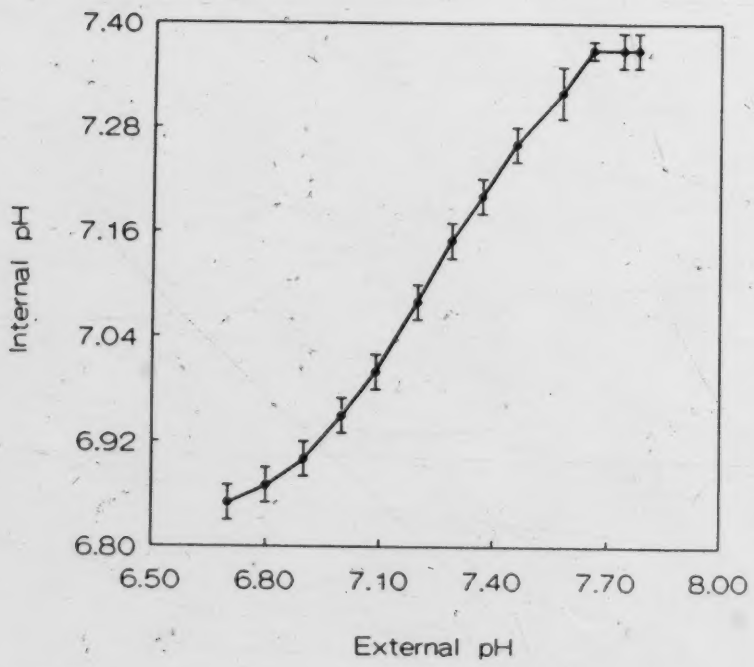

Figure 5. Effects of variation of extracellular pH (pHo) on pHi (pHi). Rat anterior pituitary cells loaded with BCECF were studied for effects of variation of pHo. The external pH was varied from 6.6 to 7.8 and the corresponding pHi was recorded. The graph (mean ± SEM) represents the relationship between pHo and pHi.

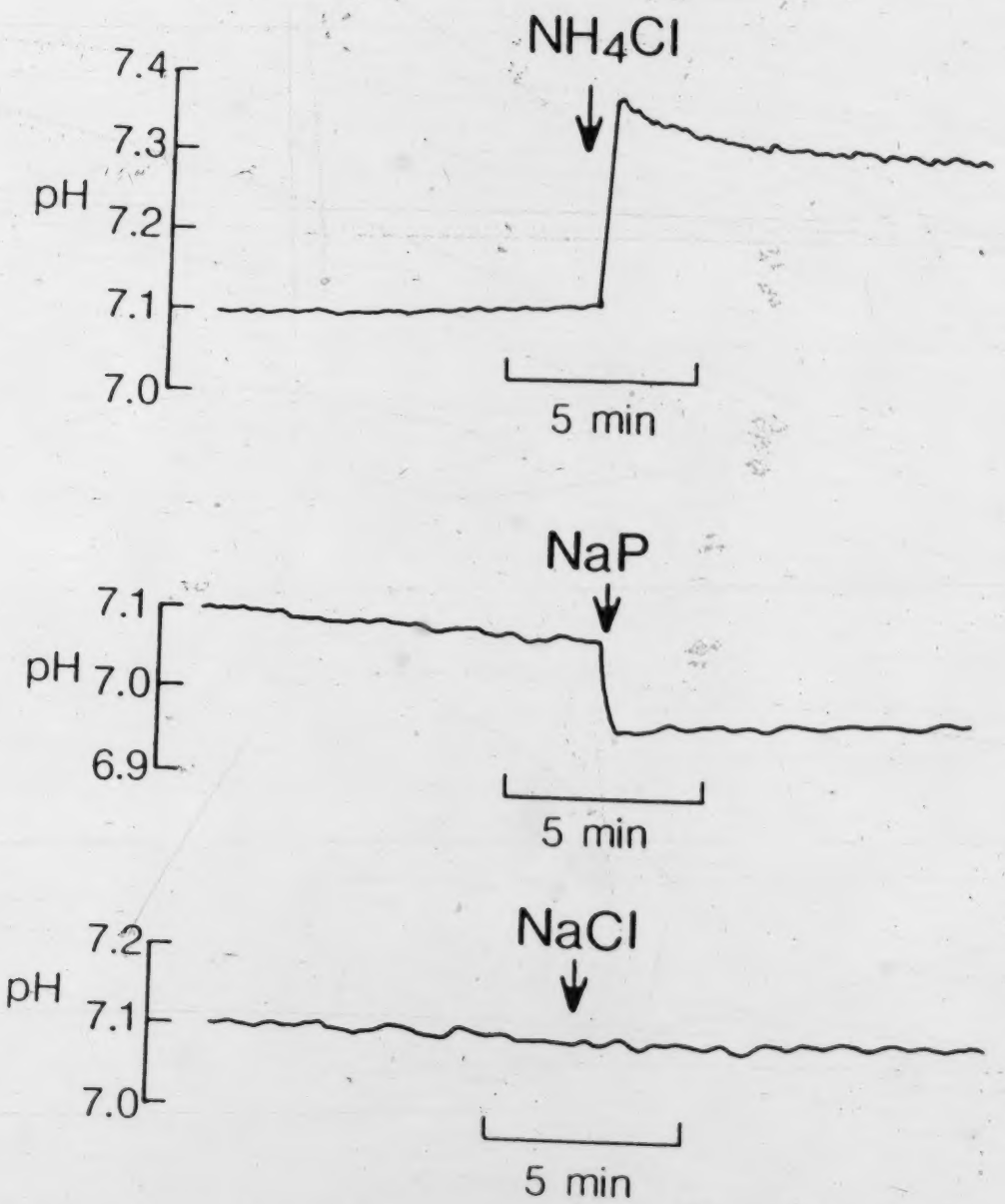

Figure 6. Effects of ammonium chloride and sodium propionate on pHi. Rat anterior cells loaded with BCECF were treated with a) ammonium chloride (NH_4Cl) (30 mM); b) sodium propionate (NaP)(30 mM); and c) sodium chloride (NaCl) (30 mM). The tracings are representative of 3 experiments.

These results indicated that inhibition of the Na^+/H^+ antiporter (using amiloride and its two derivatives), variation of pHo and ammonium chloride and sodium propionate were effective in modulating pHi in rat anterior pituitary cell cultures.

III.2. Effects of the various modulatory approaches on GHRH-stimulated cAMP release

After determining that the various modulatory approaches chosen were effective in altering pHi, the next step was to determine the effects of these approaches on GHRH-stimulated cAMP release from the anterior pituitary cells. Since a number of investigators have demonstrated that cAMP release in response to GHRH stimulation occurred in 10 to 15 min (Sheppard *et al.*, 1979), all the cAMP measurements in response to the various secretagogues were performed 15 min after stimulation.

The effect of inhibition of the Na^+/H^+ antiporter was determined using amiloride and its two potent derivatives, MIBA and DMA. At a pHo of 7.2, GHRH produced a concentration-related elevation of cAMP release more than ten-fold above basal levels. The amiloride derivative MIBA at a concentration of 10 μM significantly inhibited the GHRH-stimulated cAMP response ($P<0.005$). There was no alteration of the basal cAMP release (Figure 7). Dose-response studies demonstrated that this inhibition was dose-dependent (Figure 8). Similar dose-dependent inhibition of GHRH stimulated cAMP release was observed on treatment with DMA (Figures 9 and 10) and amiloride (Figures 11 and 12).

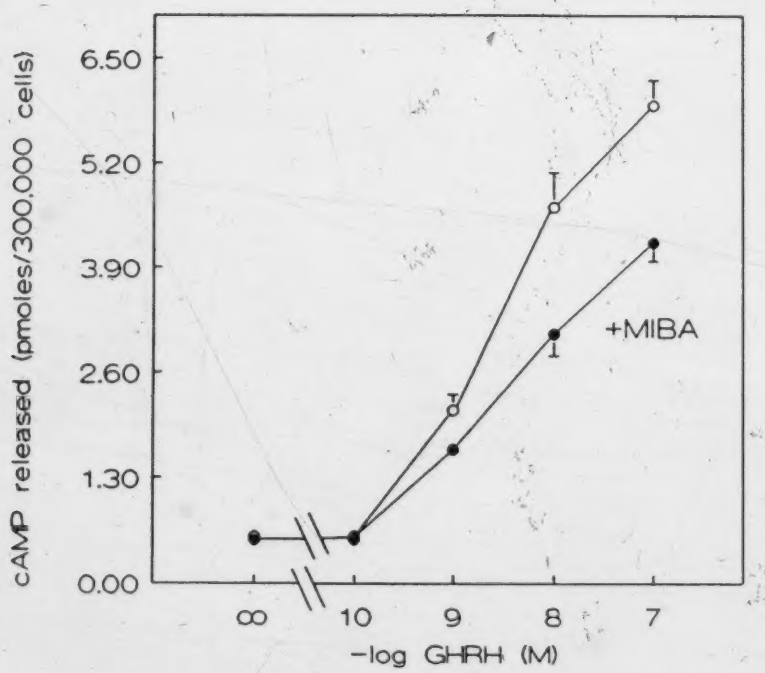

Figure 7. Effects of inhibition of the Na^+/H^+ antiporter on GHRH-stimulated cAMP release. To determine the effects of inhibition of Na^+/H^+ antiporter, the cells were pretreated with the amiloride derivative MIBA (10 μM) for 5 min. The cells were incubated with GHRH at graded concentrations in the presence or absence of MIBA. Each point represents the mean $\pm$ SEM of cAMP determinations done in duplicate on four samples of cells in three sets of experiments.

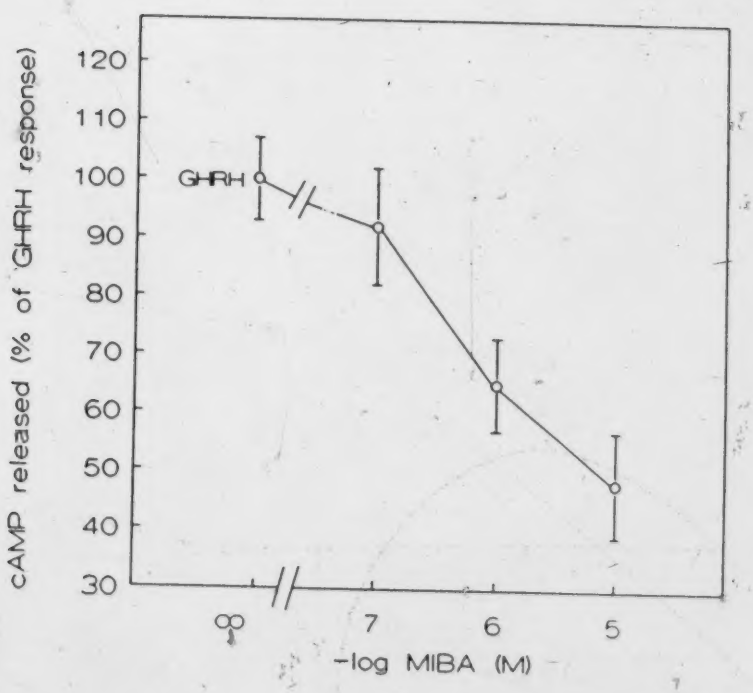

Figure 8. Dose-response of MIBA on GHRH-stimulated cAMP release. Cultured anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of MIBA. Each point represents the mean ± SEM of cAMP determinations done in duplicate on four samples of cells in three sets of experiments.

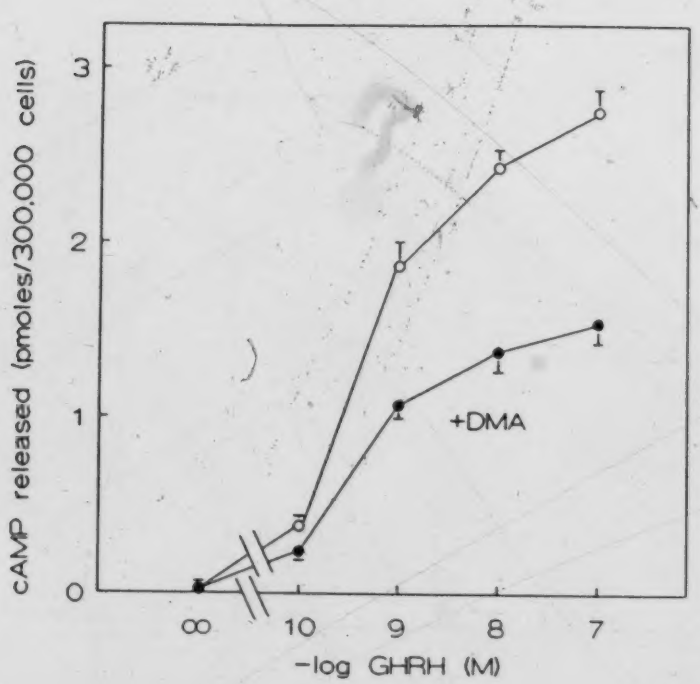

Figure 9. Effects of DMA on GHRH-stimulated cAMP release. The cells were pretreated with DMA (10 μM) for 5 min. The cells were incubated with GHRH at graded concentrations in the presence or absence of DMA. Each point represents the mean ± SEM of cAMP determinations done in duplicate on four samples of cells in three sets of experiments.

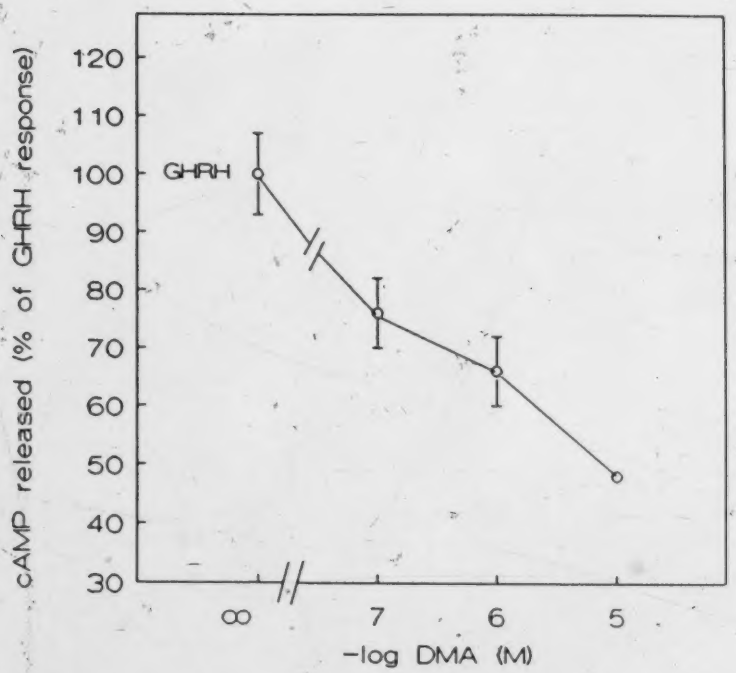

Figure 10. Dose-response of DMA on GHRH-stimulated cAMP release. Cultured anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of DMA. Each point represents the mean ± SEM of cAMP determinations done in duplicate on four samples of cells in three sets of experiments.

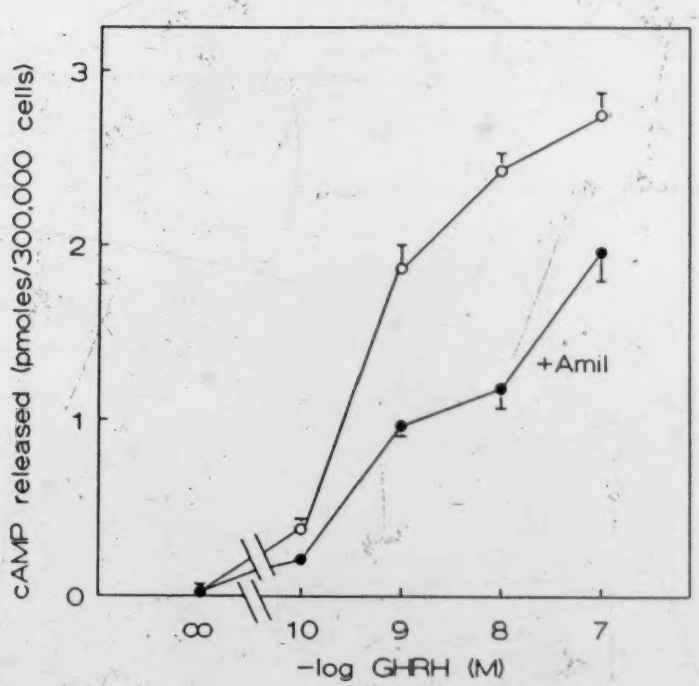

Figure 11. Effects of amiloride on GHRH-stimulated cAMP release. The cells were pretreated with amiloride (Amil) (10 μM) for 5 min. The cells were incubated with GHRH at graded concentrations in the presence or absence of amiloride. Each point represents the mean ± SEM of cAMP determinations done in duplicate on four samples of cells in three sets of experiments.

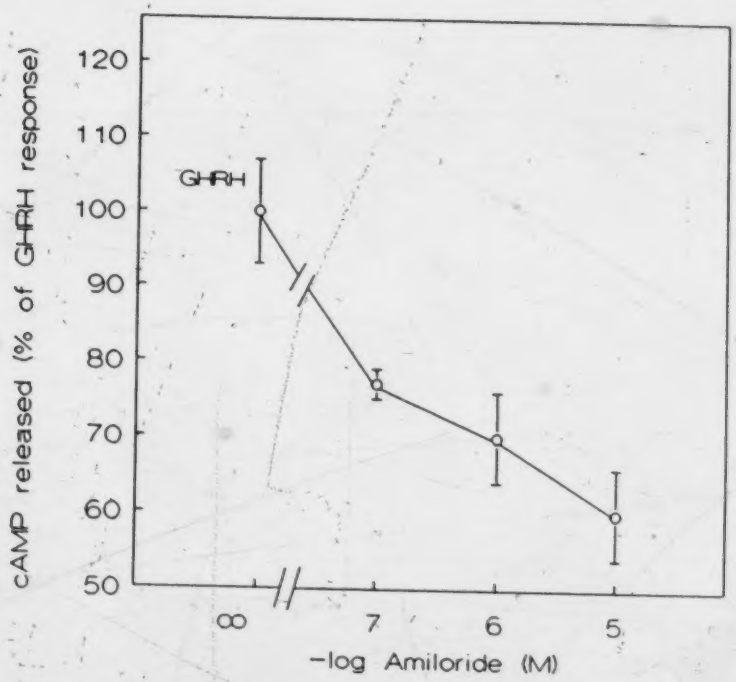

Figure 12. Dose-response of amiloride on GHRH-stimulated cAMP release. Cultured anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of amiloride. Each point represents the mean ± SEM of cAMP determinations done in duplicate on four samples of cells in three sets of experiments.

To determine the effect of varying pHo on basal and GHRH-stimulated cAMP release, the medium bathing the cells was replaced by DMEM without fetal calf serum at pH values varying from 6.6 to 7.8. GHRH was dissolved in the medium at concentrations ranging from 100 pM to 100 nM. At the end of 15 min, the medium was removed, boiled and assayed for cAMP. At all the pH values studied, GHRH stimulated cAMP release more than ten-fold above basal levels in a dose-dependent manner. When the pHo was raised from 7.4 to 7.8, GHRH-stimulated cAMP release was significantly elevated by 30% ($P<0.005$ for GHRH doses $\geq$ 1nM). When the pHo was reduced from 7.4 to 7.0, GHRH-stimulated cAMP release was significantly inhibited by 30% ($P<0.005$ for GHRH doses 1 nM and 10 nM). Further reduction of pHo inhibited GHRH-stimulated cAMP release further by 30% ($P<0.005$ for GHRH doses $\geq$ 10 nM). In all the situations, the basal cAMP release was not affected by variation in pHo (Figure 13).

The effect of reduction of pHi on GHRH-stimulated cAMP release was then studied using sodium propionate at a concentration of 30 mM. Figure 14 shows that sodium propionate had no significant effect on basal cAMP release. It, however, produced a significant inhibition of GHRH-stimulated cAMP release ($P<0.005$ for GHRH doses $\geq$ 1 nM). Dose-response studies using varying concentrations of sodium propionate demonstrated that it inhibited GHRH (10 nM)-stimulated cAMP release in a dose-dependent manner (Figure 15).

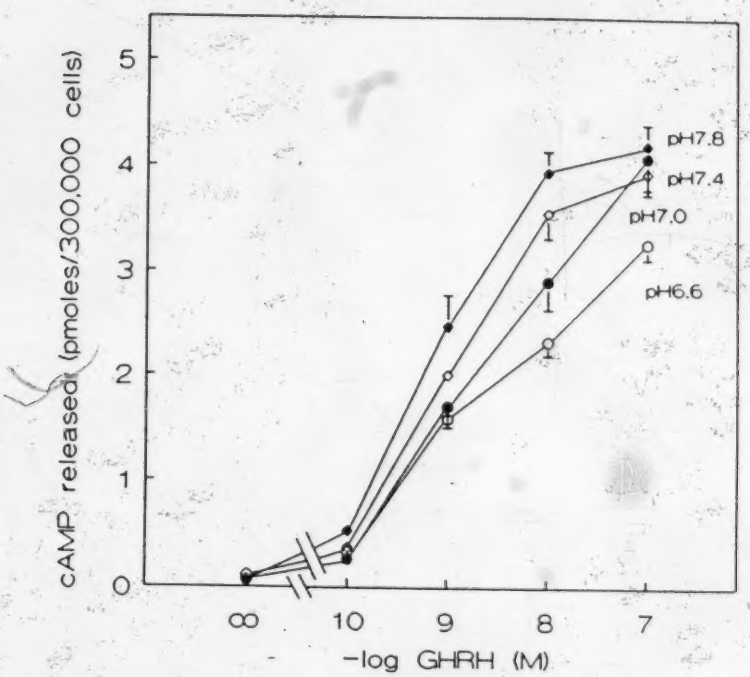

Figure 13. Effects of varying pHo on GHRH-stimulated cAMP release. The medium bathing the cells was replaced with medium at pH varying from 6.6 to 7.8. GHRH was dissolved in the medium at varying concentrations (100 pM - 100 nM). After 15 min, the medium was removed and assayed for cAMP levels. Each point represents the mean ± SEM of cAMP determinations done in duplicate on four samples of cells in three sets of experiments.

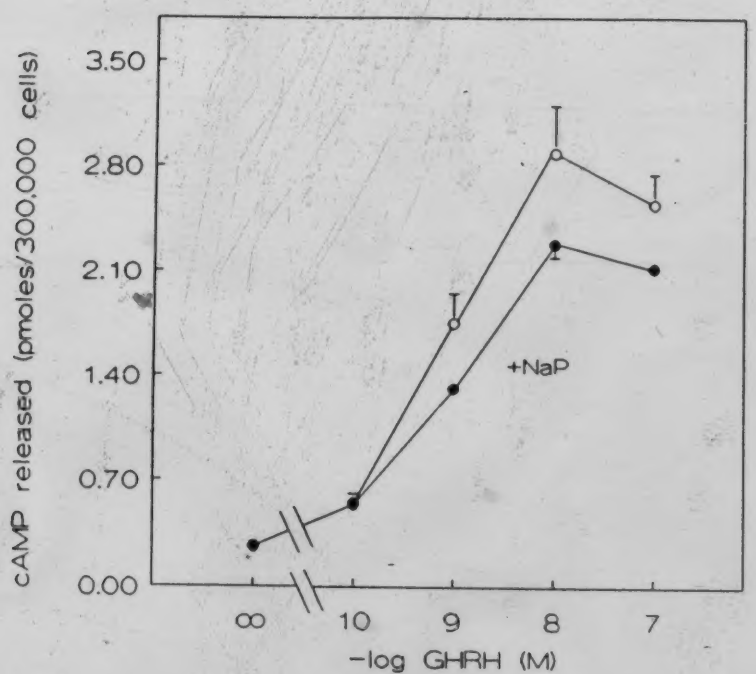

Figure 14. Effects of sodium propionate on GHRH-stimulated cAMP release. Anterior pituitary cells were incubated with GHRH at graded concentrations in the presence and absence of sodium propionate (NaP) (30 mM). Each point represents the mean ± SEM of cAMP determinations done in duplicate on four samples of cells in three sets of experiments.

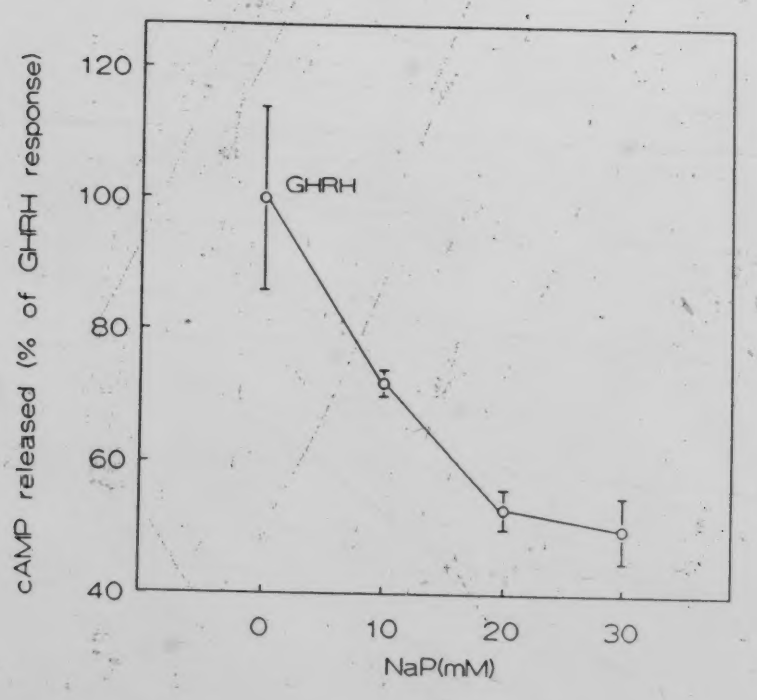

Figure 15. Dose-response of sodium propionate on GHRH-stimulated cAMP release. The anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of sodium propionate (NaP). Each point represents the mean ± SEM of cAMP determinations done in duplicate on four samples of cells in three sets of experiments.

To study the effect of elevation of pHi on GHRH-stimulated cAMP release, ammonium chloride at a concentration of 30 mM was used. Figure 16 demonstrates that ammonium chloride, while having no effect on its own, caused a twofold rise ($P<0.005$) in GHRH-stimulated cAMP release at all the concentrations of GHRH (100 pM to 100 nM) studied. This elevation in GHRH-stimulated cAMP release by ammonium chloride was also dose-dependent (Figure 17).

These results indicated that GHRH-stimulated cAMP release was directly affected by agents shown to modulate pHi; agents shown to elevate pHi produced a significant elevation of GHRH-stimulated cAMP release whereas agents which reduce pHi produced a significant inhibition of GHRH-stimulated cAMP release. In contrast, basal cAMP release was not influenced by changes in pHi.

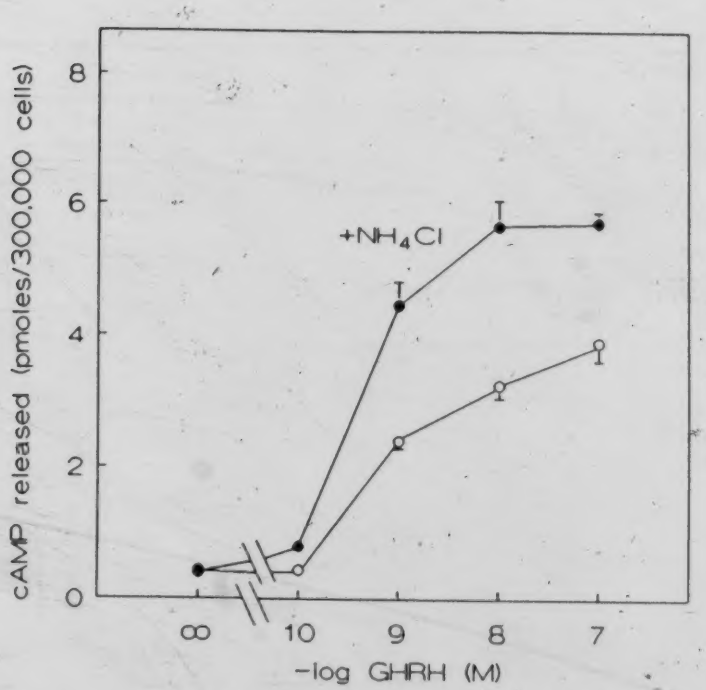

Figure 16. Effects of ammonium chloride on GHRH-stimulated cAMP release. Anterior pituitary cells were incubated with GHRH at graded concentrations in the presence or absence of ammonium chloride (NH_4Cl) (30 mM). Each point represents the mean ± SEM of cAMP determinations done in duplicate on four samples of cells in three sets of experiments.

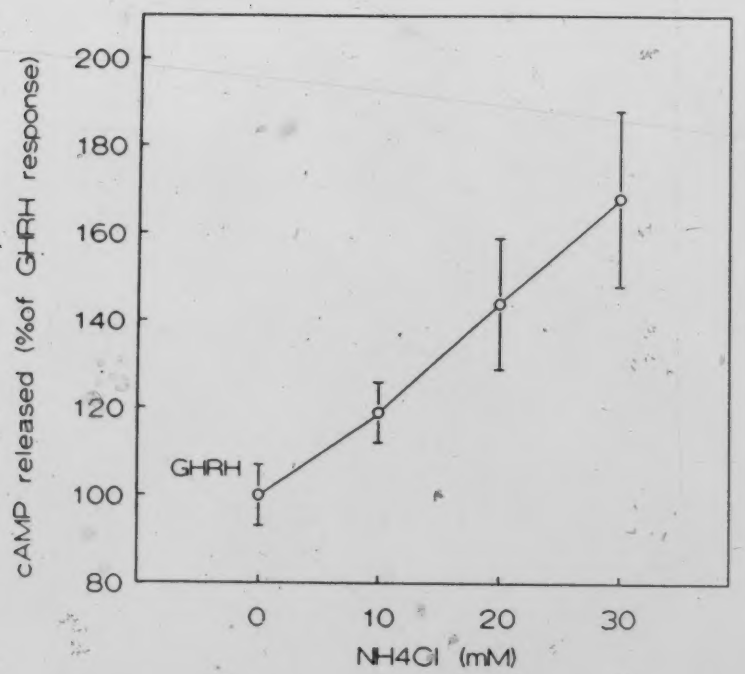

Figure 17. Dose-response of ammonium chloride on GHRH-stimulated cAMP release. Cultured anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of ammonium chloride (NH_4Cl). Each point represents the mean ± SEM of cAMP determinations done in duplicate on four samples of cells in three sets of experiments.

III.3 Effects of the various modulatory approaches on GHRH-stimulated GH release 15 minutes after stimulation

The effects of experimental modulation of pHi on GH secretion were also studied using the same three approaches used above. To study the effects on the early and late release pools of GH, the release of GH in response to the various experimental approaches was done at two time points: 15 min and 2 h after stimulation.

At the same concentrations studied for cAMP release, GHRH produced a dose-dependent stimulation of GH release above basal levels. Pretreatment with the Na^+/H^+ antiporter inhibitor MIBA at a concentration of 10 μM, inhibited the GHRH-stimulated GH release by 40% ($P<0.005$) at GHRH concentrations of 10 and 100 nM and had no significant effect on basal GH release (Figure 18). Significant inhibition was observed with 0.1 μM of MIBA (Figure 19). Pretreatment with the amiloride derivative DMA at a concentration of 10 μM also inhibited GHRH-stimulated GH release by 50% ($P<0.005$) as demonstrated in Figure 20. This inhibition was dose-dependent (Figure 21). Amiloride also inhibited GHRH-stimulated GH release by 30% (Figure 22) in a dose-dependent manner (Figure 23).

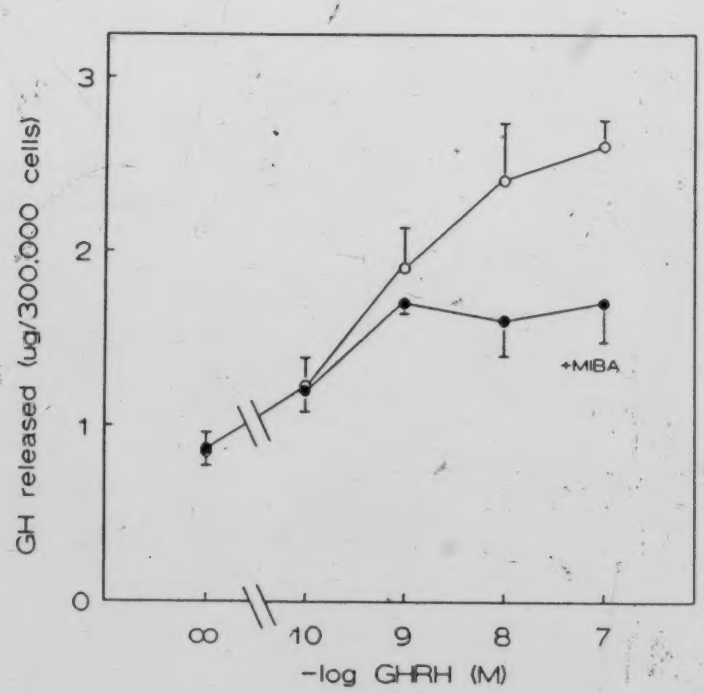

Figure 18. Effects of inhibition of the Na^+/H^+ antiporter on GH release (15 min post GHRH stimulation). The cells were incubated with GHRH at graded concentrations in the presence or absence of MIBA (10 μM). Each point represents the mean $\pm$ SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

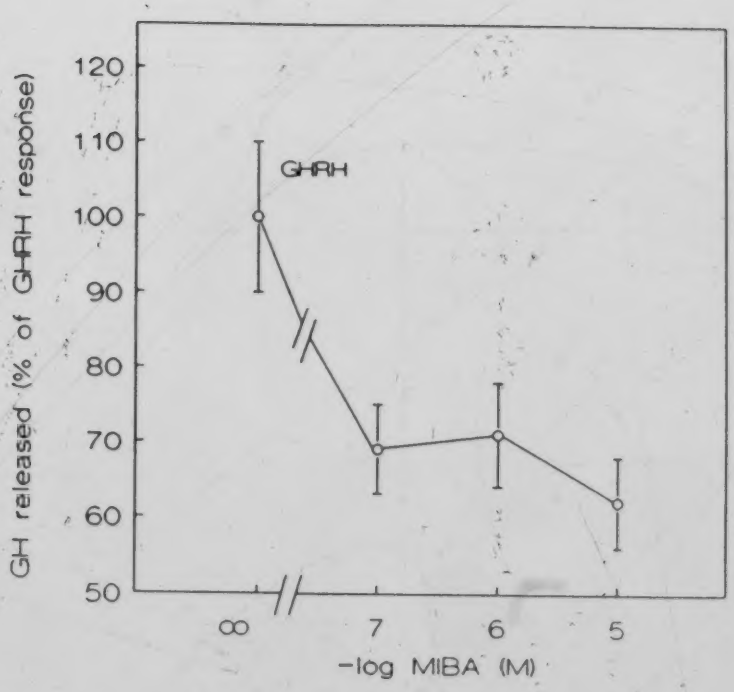

Figure 19. Dose-response of MIBA on GH release (15 min post GHRH stimulation). Cultured anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of MIBA. Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

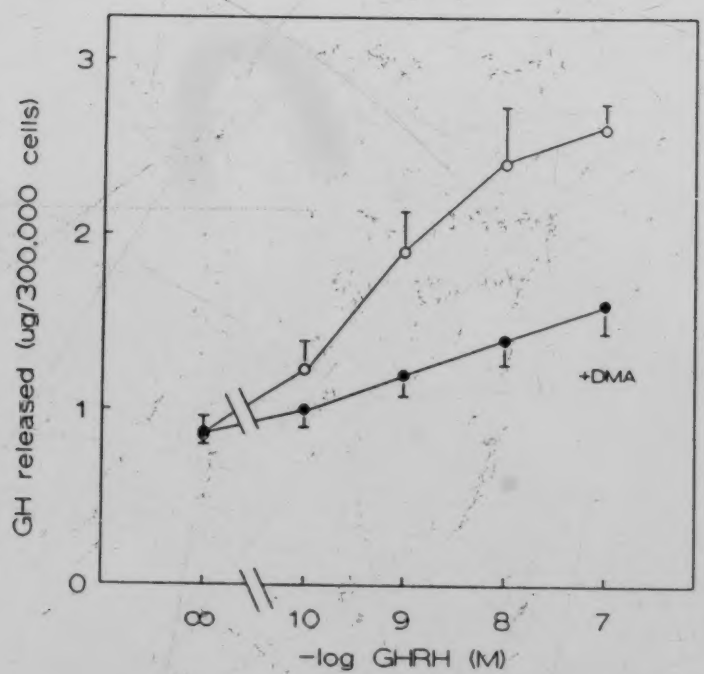

Figure 20. Effects of DMA on GH release (15 min post GHRH stimulation). The cells were incubated with GHRH at graded concentrations in the presence or absence of DMA (10 μM). Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

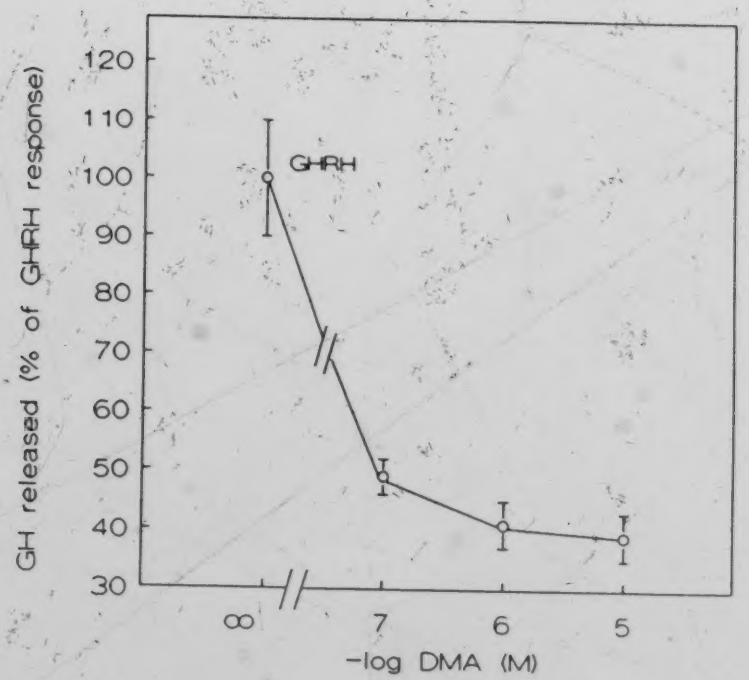

Figure 21. Dose-response of DMA on GH release (15 min post GHRH stimulation). Cultured anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of DMA. Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

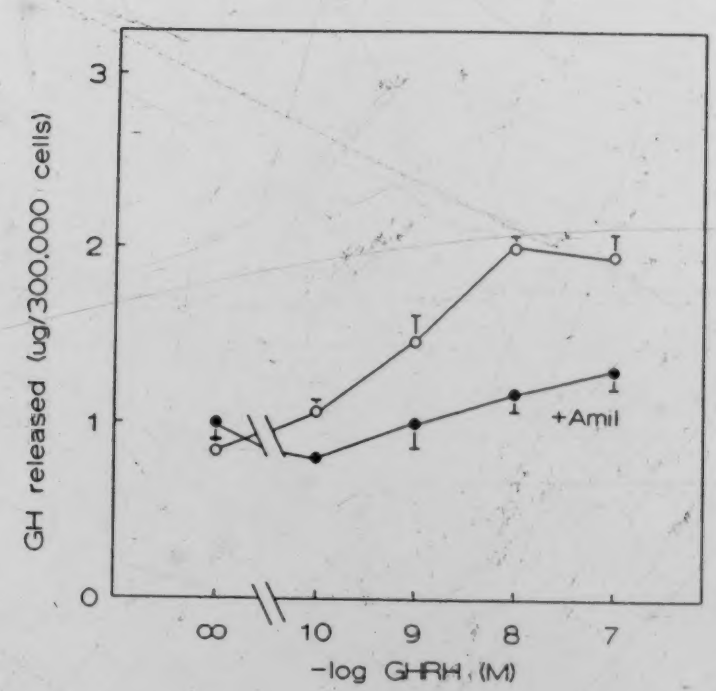

Figure 22. Effects of amiloride on GH release (15 min post GHRH stimulation). The cells were incubated with GHRH at graded concentrations in the presence or absence of amiloride (Amil) (10 μM). Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

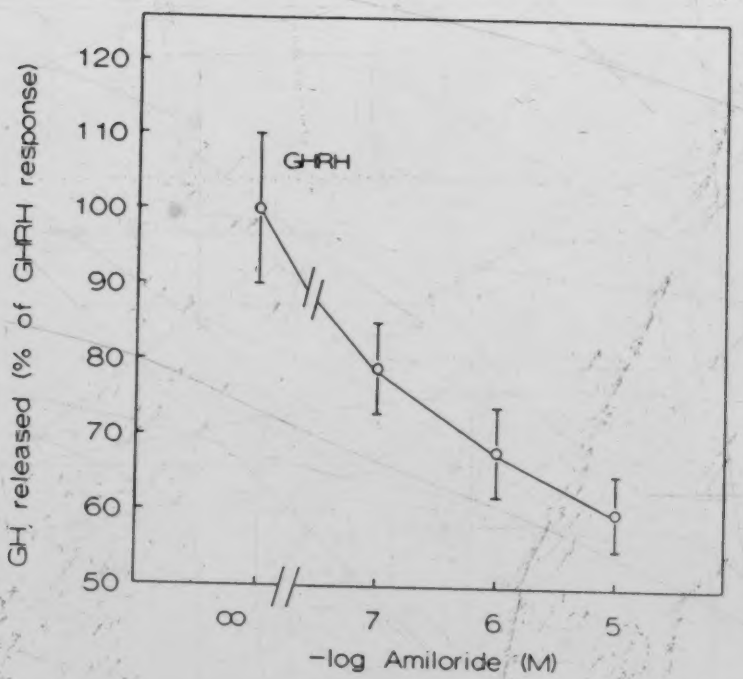

Figure 23. Dose-response of amiloride on GH release (15 min post GHRH stimulation). Cultured anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of amiloride. Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

Figure 24 demonstrates the effect of varying pHo on basal and GHRH-stimulated GH release. At all the pHo values, GHRH produced a dose-dependent stimulation of GH release above basal levels. An increase in pHo from 6.6 to 7.0 had no significant effect on basal or GHRH-stimulated GH release ($P>0.05$ for all GHRH doses tested). However, an increase from 7.0 to 7.8 raised the basal GH release six-fold ($P<0.005$). In addition, this increase in pHo also resulted in an augmented GHRH-stimulated GH release ($P<0.05$ for all GHRH doses tested).

The effects of reduction of pHi on GH release was then determined using sodium propionate. At a concentration of 30 mM, sodium propionate also produced a significant inhibition ($P<0.005$) of GHRH-stimulated GH release. However, it had no effect on basal GH release (Figure 25). This inhibition by sodium propionate was seen at all the doses studied (Figure 26).

The effects of elevation of pHi on GH release was studied using ammonium chloride. At a concentration of 30 mM, ammonium chloride had little effect on basal GH release. Surprisingly, ammonium chloride produced a significant reduction ($P<0.005$) of GHRH-stimulated GH release (Figure 27). This inhibition by ammonium chloride was also dose-dependent (Figure 28).

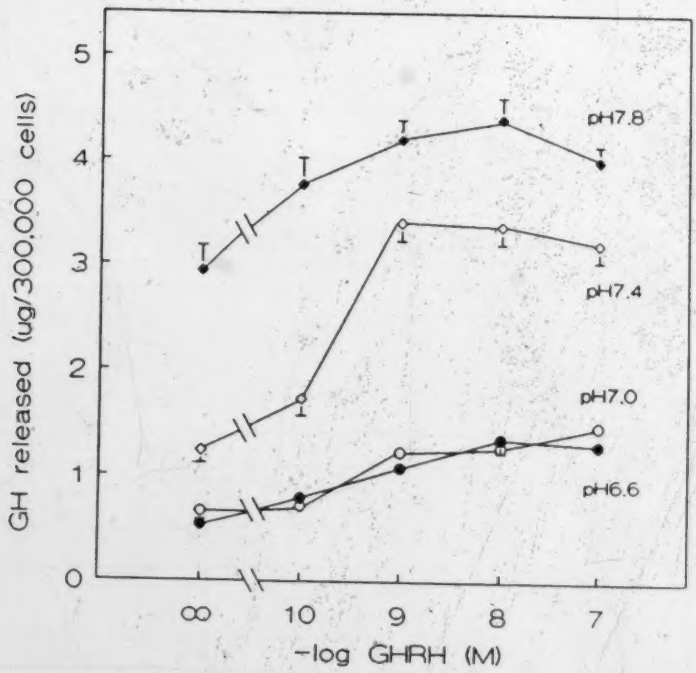

Figure 24. Effects of varying pHo on GH release (15 min post GHRH stimulation). The medium bathing the cells was replaced with medium at pH varying from 6.6 to 7.8. GHRH was dissolved in the medium at varying concentrations (100 pM - 100 nM). After 15 min, the medium was removed and assayed for GH levels. Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells.

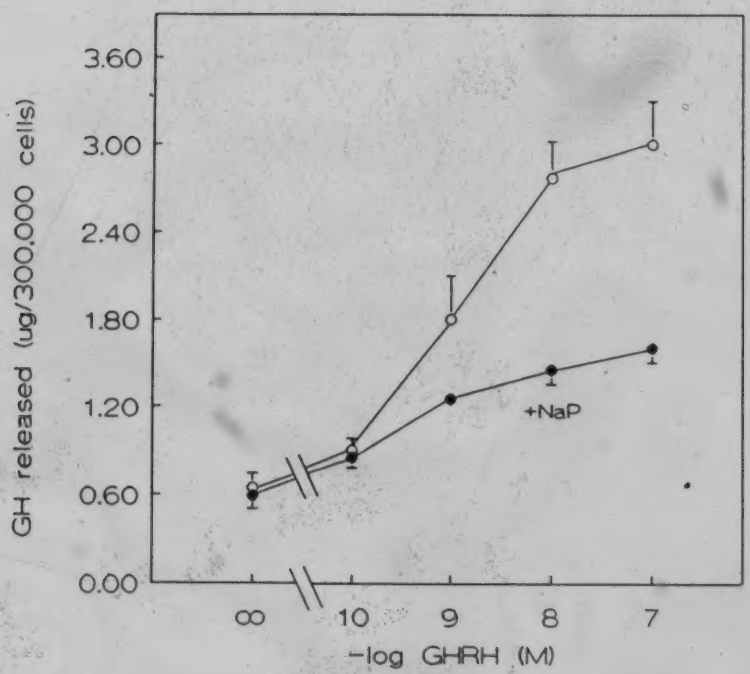

Figure 25. Effects of sodium propionate on GH release (15 min post GHRH stimulation). Anterior pituitary cells were incubated with GHRH at graded concentrations in the presence or absence of sodium propionate (NaP) (30 mM). Each point represents the mean ± SEM of GH deter-minations done in duplicate on four samples of cells in three sets of experiments.

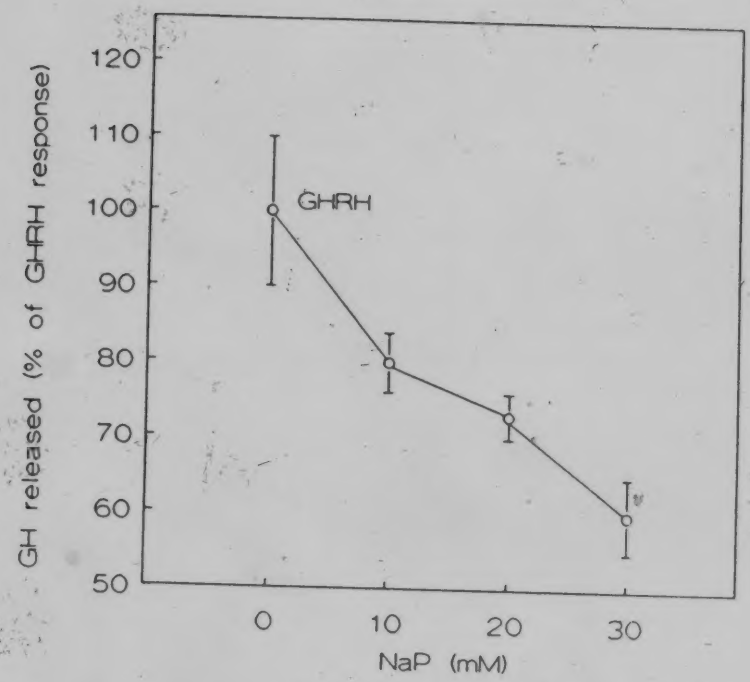

Figure 26. Dose-response of sodium propionate on GH release (15 min post GHRH stimulation). The anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of sodium propionate (NaP). Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

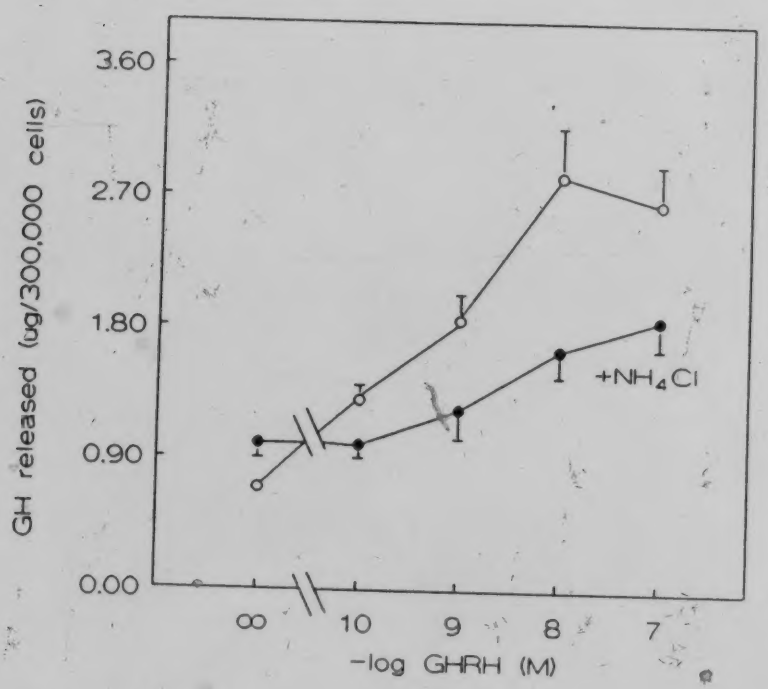

Figure 27. Effects of ammonium chloride on GH release (15 min post GHRH stimulation). Anterior pituitary cells were incubated with GHRH at graded concentrations in the presence or absence of ammonium chloride (NH_4Cl) (30 mM). Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

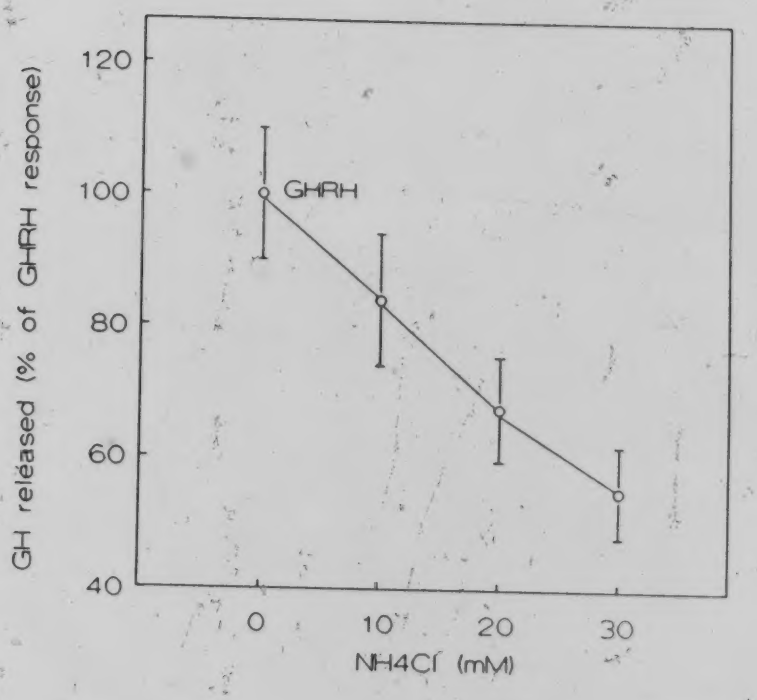

Figure 28. Dose-response of ammonium chloride on GH release (15 min post GHRH stimulation). Cultured anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of ammonium chloride (NH_4Cl). Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

III.4 Effects of the various modulatory approaches on GHRH-stimulated GH release two hours after stimulation

For the study of the release of the late pool of GH, the cells were treated as described above. After 15 min, the medium was removed to eliminate the early release pool and replaced with fresh medium containing the same secretagogues. Two hours after the initial stimulation the medium was removed and assayed for GH release by RIA.

GHRH produced a dose-dependent stimulation of GH release above basal levels 2 h after stimulation. Pretreatment with the Na^+/H^+ antiporter inhibitor MIBA at a concentration of 10 μM inhibited the GHRH-stimulated GH release by 40% ($P<0.005$) only at GHRH concentrations of 10 nM and 100 nM, without having any significant effect on basal GH release (Figure 29). This inhibition was seen only at a dose of 10 μM of MIBA (Figure 30). Pretreatment with the amiloride derivative DMA at a concentration of 10 μM also inhibited GHRH-stimulated GH release by 50% ($P<0.005$) at higher concentrations of GHRH as demonstrated in Figure 31. This inhibition was seen at all doses studied (Figure 32). Amiloride had a small inhibitory effect (by 15%) ($P<0.005$ for GHRH doses of 10 nM and 100 nM) on GHRH-stimulated GH release (Figure 33). No dose-dependency could be demonstrated (Figure 34).

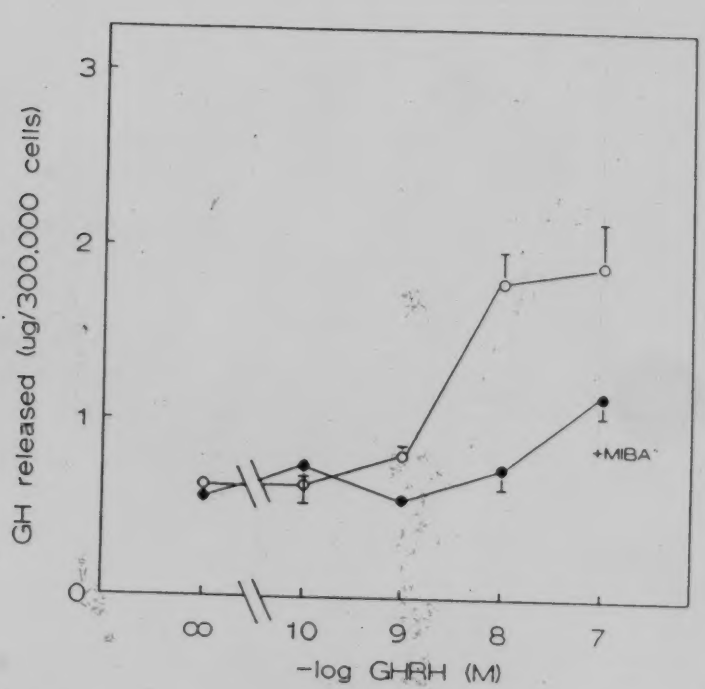

Figure 29. Effects of inhibition of the Na^+/H^+ antiporter on GH release (2 h post GHRH stimulation). The cells were incubated with GHRH at graded concentrations in the presence or absence of MIBA (10 μM). Each point represents the mean $\pm$ SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

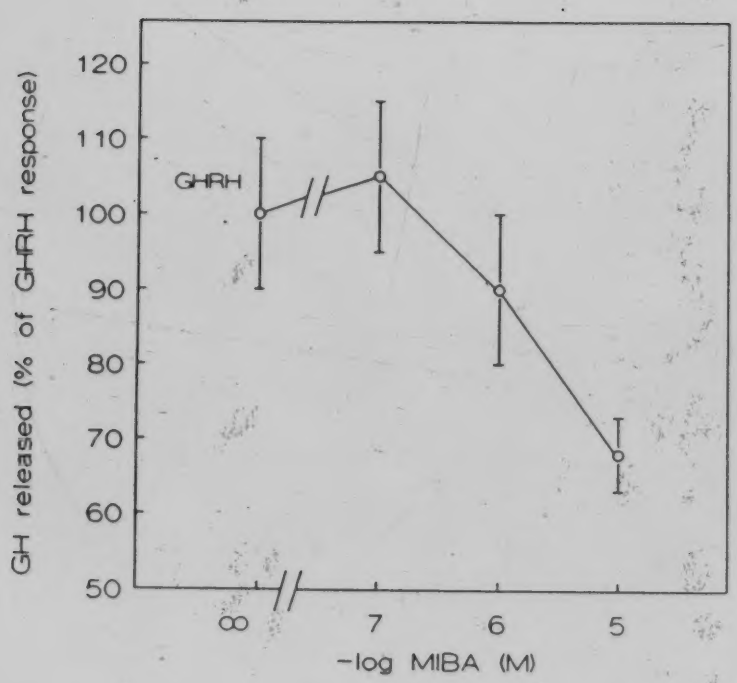

Figure 30. Dose-response of MIBA on GH release (2 h post GHRH stimulation). Cultured anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of MIBA. Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

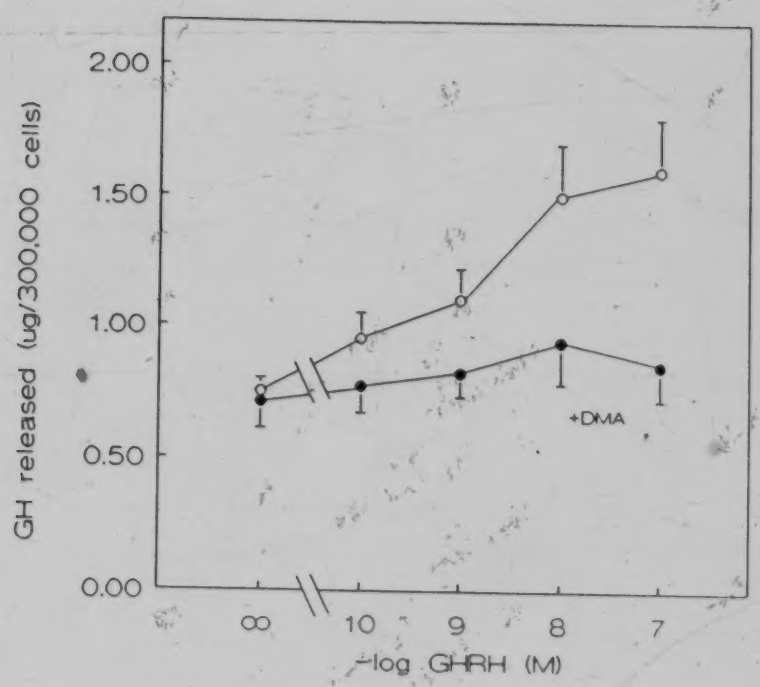

Figure 31. Effects of DMA on GH release (2 h post GHRH stimulation). The cells were incubated with GHRH at graded concentrations in the presence or absence of DMA (10 μM). Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

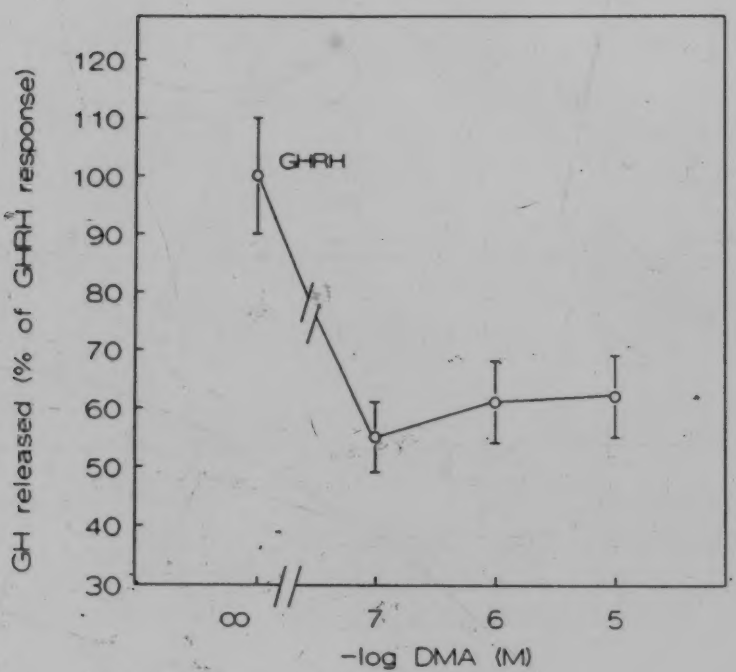

Figure 32. Dose-response of DMA on GH release (2 h post GHRH stimulation). Cultured anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of DMA. Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

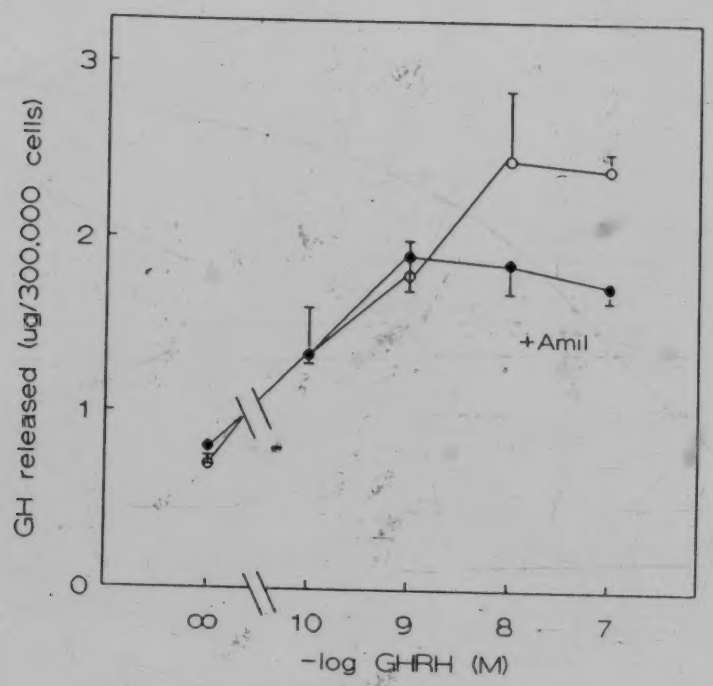

Figure 33. Effects of amiloride on GH release (2 h post GHRH stimulation). The cells were incubated with GHRH at graded concentrations in the presence or absence of amiloride (Amil) (10 μM). Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

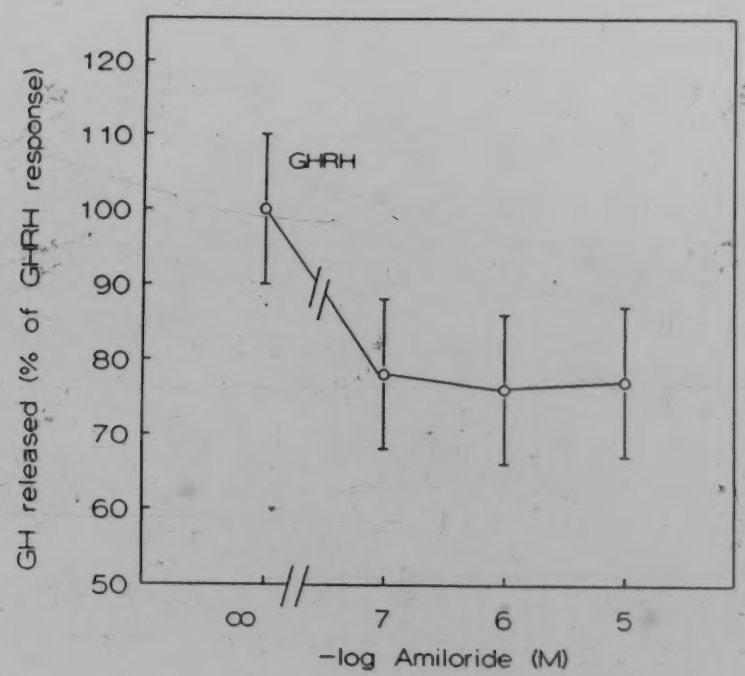

Figure 34. Dose-response of amiloride on GH release (2 h post GHRH stimulation). Cultured anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of amiloride. Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

Figure 35 demonstrates the effect of varying pHo on basal and GHRH-stimulated GH release. At both the pHo values studied (7.0 and 7.7), GHRH produced a dose-dependent stimulation of GH release above basal levels. An increase in external pH from 7.0 to 7.7 raised the basal GH release three-fold ($P<0.005$). GHRH-stimulated GH release was also significantly elevated at a lower concentration of GHRH (100 pM) but maximal GHRH-stimulated GH release was not significantly different.

The effects of reduction of pHi on GH release was then determined using sodium propionate. At a concentration of 30 mM, sodium propionate also produced a significant inhibition ($P<0.005$) of GHRH-stimulated GH release. However, it had no effect on basal GH release (Figure 36). This inhibition by sodium propionate was dose-dependent (Figure 37).

The effects of elevation of pHi on GH release was studied using ammonium chloride. At a concentration of 30 mM, ammonium chloride had little effect on basal GH release. However, ammonium chloride produced a significant reduction ($P<0.005$) of GHRH-stimulated GH release (Figure 38). This inhibition by ammonium chloride was seen at all doses studied (Figure 39).

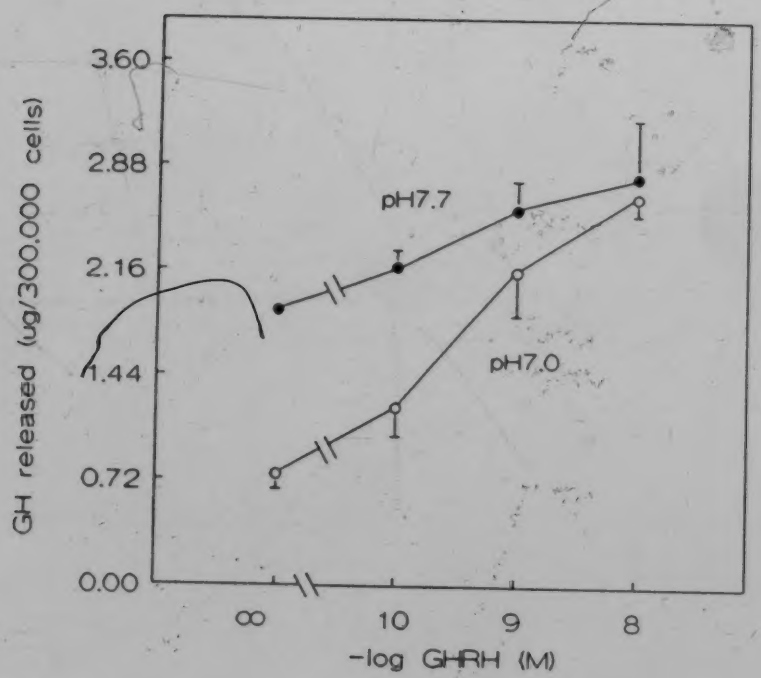

Figure 35. Effects of varying pHo on GH release (2 h post GHRH stimulation). The medium bathing the cells was replaced with medium at pHo 7.0 and 7.7. GHRH was dissolved in the medium at varying concentrations (100 pM-10 nM). After two hours, the medium was removed and assayed for GH levels. Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells.

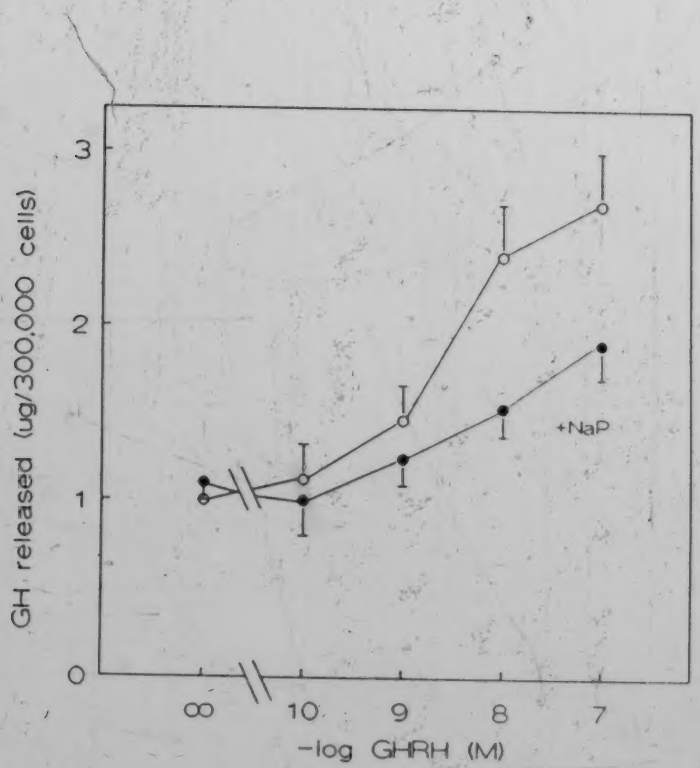

Figure 36. Effects of sodium propionate on GH release (2 h post GHRH stimulation). Anterior pituitary cells were incubated with GHRH at graded concentrations in the presence or absence of sodium propionate (NaP) (30 mM). Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

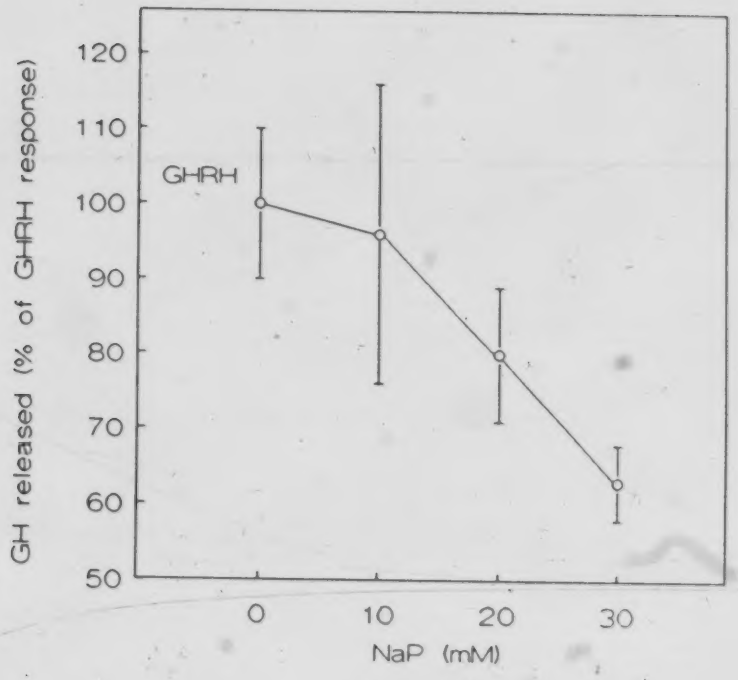

Figure 37. Dose-response of sodium propionate on GH release (2 h post GHRH stimulation). The anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of sodium propionate (NaP). Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

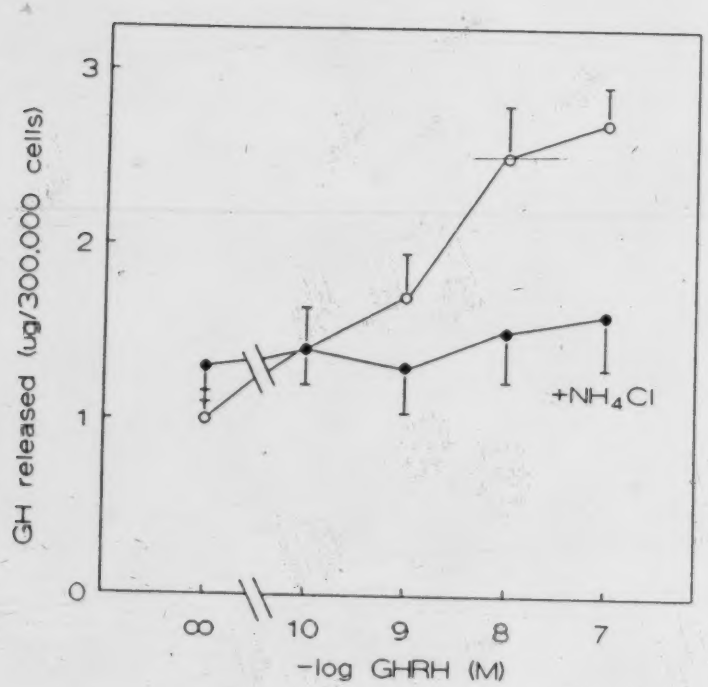

Figure 38. Effects of ammonium chloride on GH release (2 h post GHRH stimulation). Anterior pituitary cells were incubated with GHRH at graded concentrations in the presence or absence of ammonium chloride (NH_4Cl) (30 mM). Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

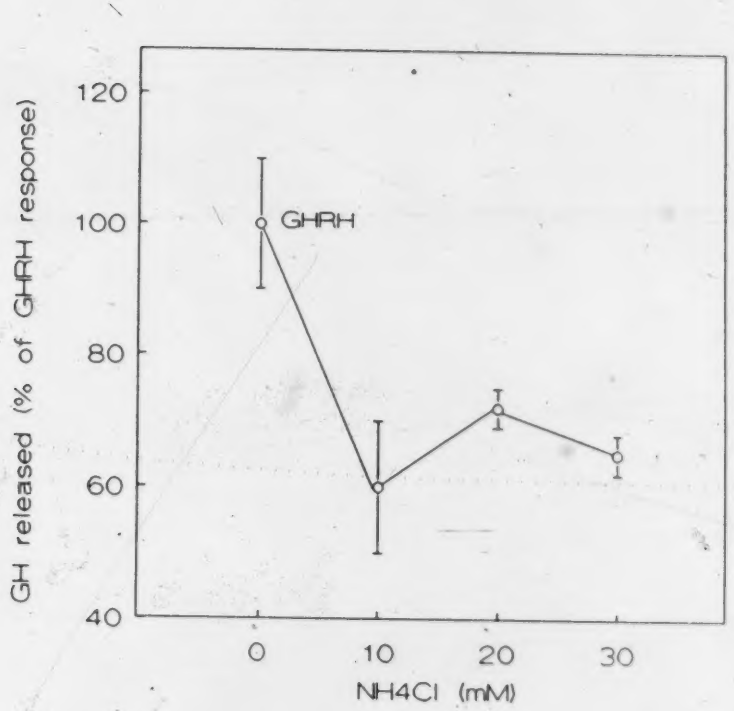

Figure 39. Dose-response of ammonium chloride on GH release (2 h post GHRH stimulation). Cultured anterior pituitary cells were incubated with GHRH (10 nM) in the presence or absence of graded concentrations of ammonium chloride (NH_4Cl). Each point represents the mean ± SEM of GH determinations done in duplicate on four samples of cells in three sets of experiments.

III. 5 Effects of the modulatory approaches on intracellular Ca^{2+} ($[Ca^{2+}]i$)

The studies on cAMP and GH release have demonstrated that reduction of pHi through inhibition of the Na^+/H^+ antiporter, reducing pHo or through sodium propionate significantly reduced GHRH-stimulated cAMP release. In comparison, elevation of pHi through elevation of extracellular pH or with ammonium chloride significantly elevated GHRH-stimulated cAMP release.

This parallel relationship between pHi and GHRH-stimulated cAMP release was, however, not observed between pHi and GH release. Reduction of pHi through the inhibition of the Na^+/H^+ antiporter using amiloride and its two derivatives, reduction of pHo or through sodium propionate significantly inhibited GHRH-stimulated GH release, an effect similar to the effects on GHRH-stimulated cAMP release. The effects of elevation of pHi on GH release were, however, more complicated suggesting that the effects of pHi on GH release may not entirely reflect its action on the cAMP pathway. Elevation of pHi by elevating extracellular pH produced a three-fold elevation of basal GH release and a small elevation of GHRH-stimulated GH release. However, elevation of pHi using ammonium chloride had little effect on basal GH release but significantly inhibited GHRH-stimulated GH release. The opposite effects of elevation of pHi by the two different approaches indicate that more than one pathway involved in GH

secretion may be sensitive to changes in pHi.

Since the other major pathway postulated to be involved in GH secretion is the Ca^{2+} pathway, a modulatory effect of pHi on this pathway may help explain some of the inconsistent effects on GH release. With this possibility in mind, the effects of the various modulatory approaches on basal and GHRH-stimulated $[Ca^{2+}]i$ were determined.

Many investigators have demonstrated that GHRH produces elevation of $[Ca^{2+}]i$ in dispersed pituitary cells (Lussier *et al.*, 1991). This was confirmed in the present study by loading the pituitary cells with the fluorescent indicator fura 2-AM and monitoring the excitation wavelengths at 380 and 340 nm and the emission wavelength at 510 nm (Materials and Methods). The values for $[Ca^{2+}]i$ obtained were lower than those demonstrated by Lussier *et al.* but could be explained by the fact that the cells used were a mixed cell population with the somatotrophs accounting for only 50%.

After the baseline of $[Ca^{2+}]i$ stabilised (137 $\pm$ 10 nM), the cells were stimulated with GHRH. The tracing (Figure 40) demonstrates that GHRH produced a dose-dependent elevation of $[Ca^{2+}]i$ up to 210 $\pm$ 13 nM (n=6).

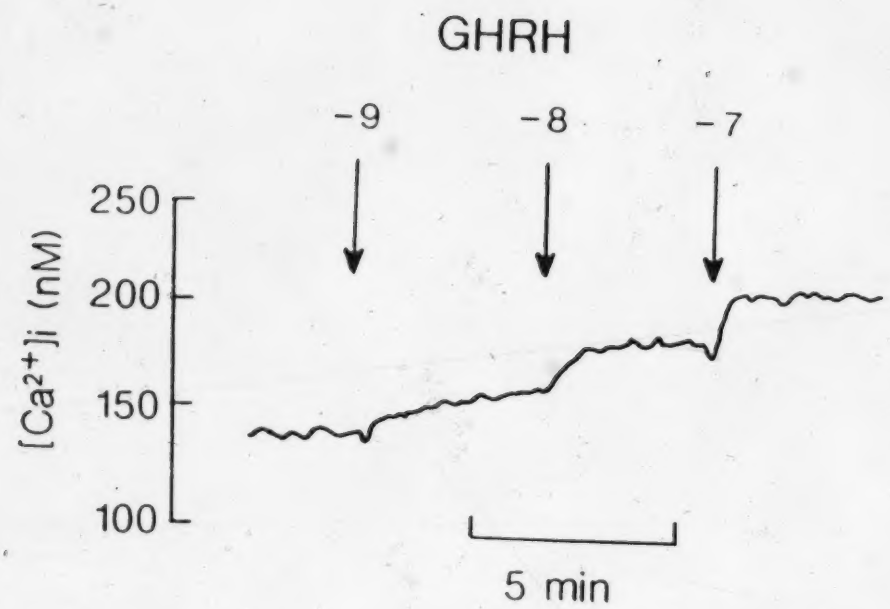

Figure 40. Effects of GHRH on $[Ca^{2+}]i$. Rat anterior pituitary cells were prepared and loaded with the $Ca2^{+}$ indicator Fura-2. Ratio of the fluorescence emission signal at 510 nm, excited at 340 and 380 nm was continuously recorded and calibrated as described. After stabilisation of the baseline, GHRH (10^{-9} M - 10^{-7} M) was added and the emission signal was monitored. The trace is representative of three experiments.

To help explain the effects of variation of pHo on GH release, the effect of this variation on $[Ca^{2+}]i$ was determined. At a pHo of 7.2, $[Ca^{2+}]i$ stabilised at 142 ± 10 nM. Acute elevation of pHo to 7.8 produced a rapid elevation of basal $[Ca^{2+}]i$ to 212 ± 15 nM (Figure 41). Subsequent addition of GHRH produced a significant though smaller increase in $[Ca^{2+}]i$ (228 ± 18 nM). To study the effects of acute elevation of pHo on GHRH-stimulated $[Ca^{2+}]i$, the cells were first treated with GHRH at a concentration of 100 nM. GHRH elevated basal $[Ca^{2+}]i$ from 128 ± 8 nM to [illegible] ± 10 nM. Elevation of pHo further elevated the $[Ca^{2+}]i$ to 222 ± 17 nM (Figure 41).

Reduction of pHo by HCl did not produce any significant effect on basal $[Ca^{2+}]i$ levels. However, following the reduction of pHo, GHRH-stimulated $[Ca^{2+}]i$ response was smaller (148 ± 12 nM to 172 ± 10 nM). Addition of HCl following GHRH had little effect on the GHRH-stimulated $[Ca^{2+}]i$ response (Figure 42).

In comparison, reduction of pHi using sodium propionate resulted in a small decrease in basal $[Ca^{2+}]i$ levels from 140 ± 15 nM to 120 ± 10 nM (Figure 44). However, subsequent addition of GHRH remained effective in elevating $[Ca^{2+}]i$. Treatment with sodium propionate also resulted in a drop of GHRH-stimulated $[Ca^{2+}]i$ levels from 190 ± 4 nM to 160 ± 9 nM (Figure 44).

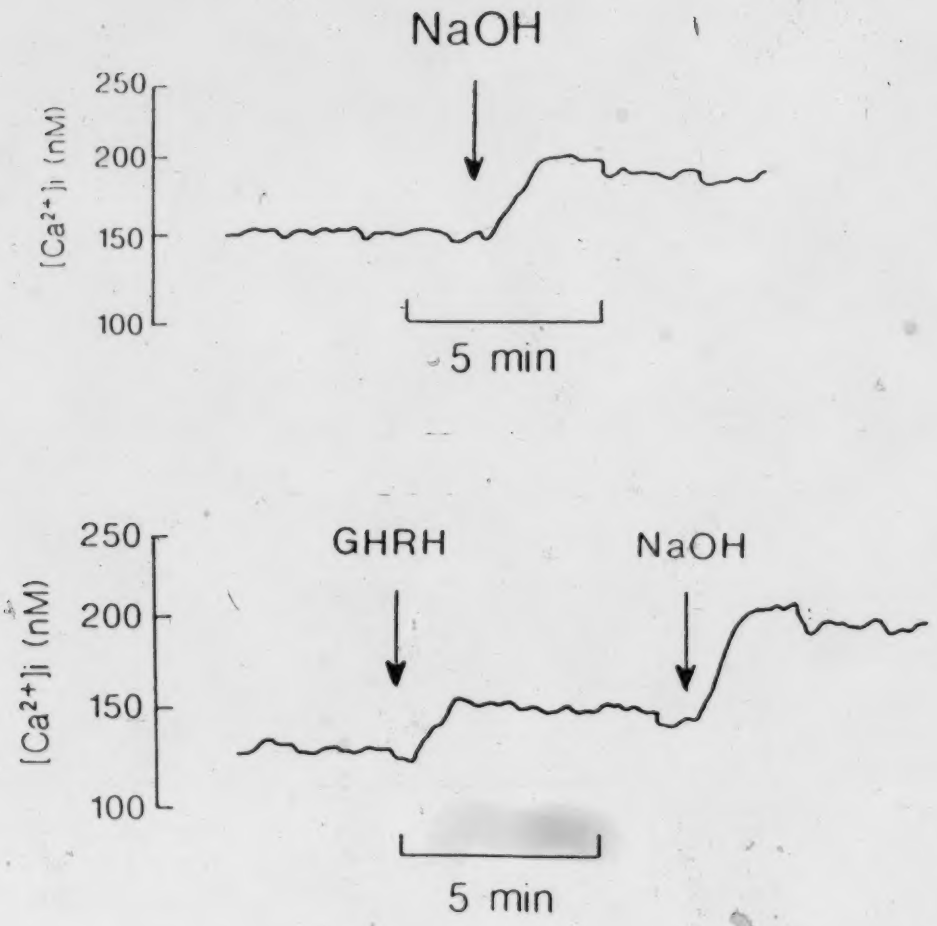

Figure 41. Effects of acute elevation of pHo on $[Ca^{2+}]i$. After stabilisation of the baseline, NaOH (5 mM) was added (pH changed from 7.2-7.8) to determine the effects on a) basal and b) GHRH-stimulated $[Ca^{2+}]i$. The tracing is representative of three experiments.

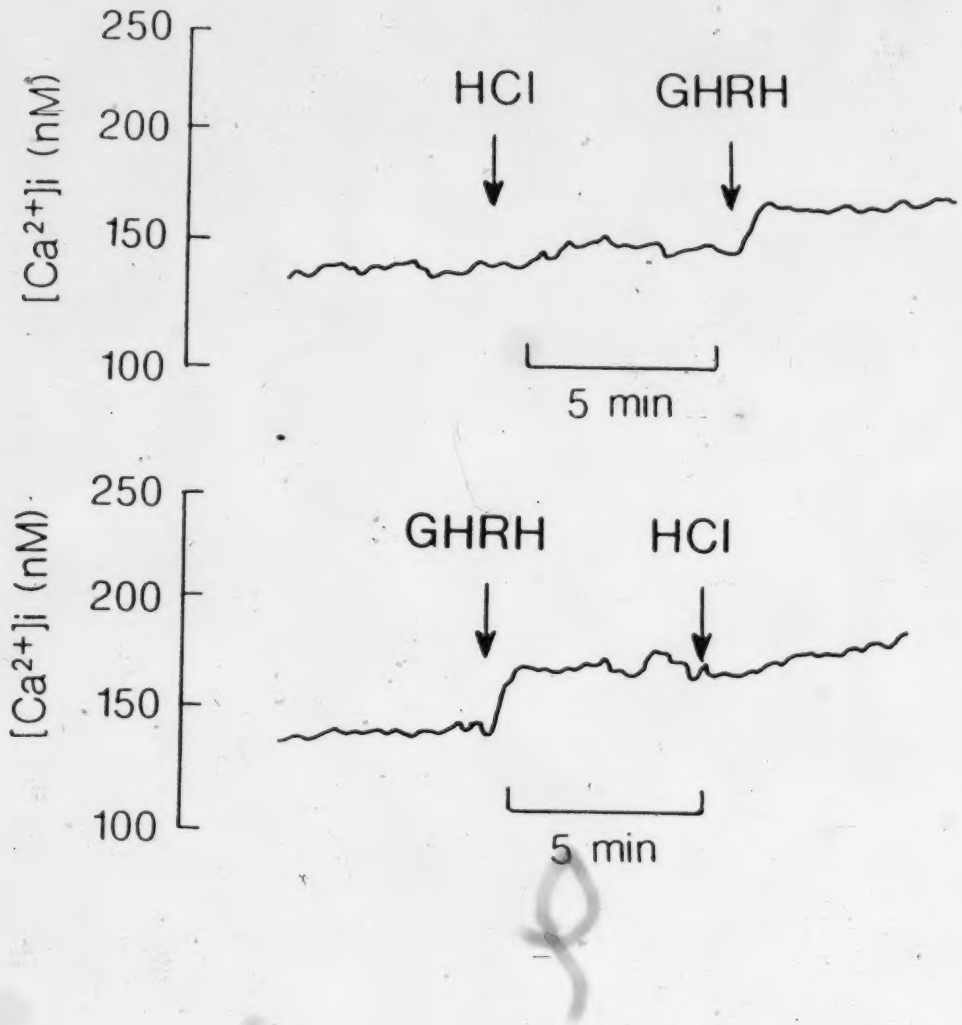

Figure 42. Effects of acute reduction of pHo on $[Ca^{2+}]i$. The effect of reduction of pHo on a) basal and b) GHRH-stimulated $[Ca^{2+}]i$ was determined using 5 mM HCl which reduces pHo from 7.2 to 6.6. The tracing is representative of three experiments.

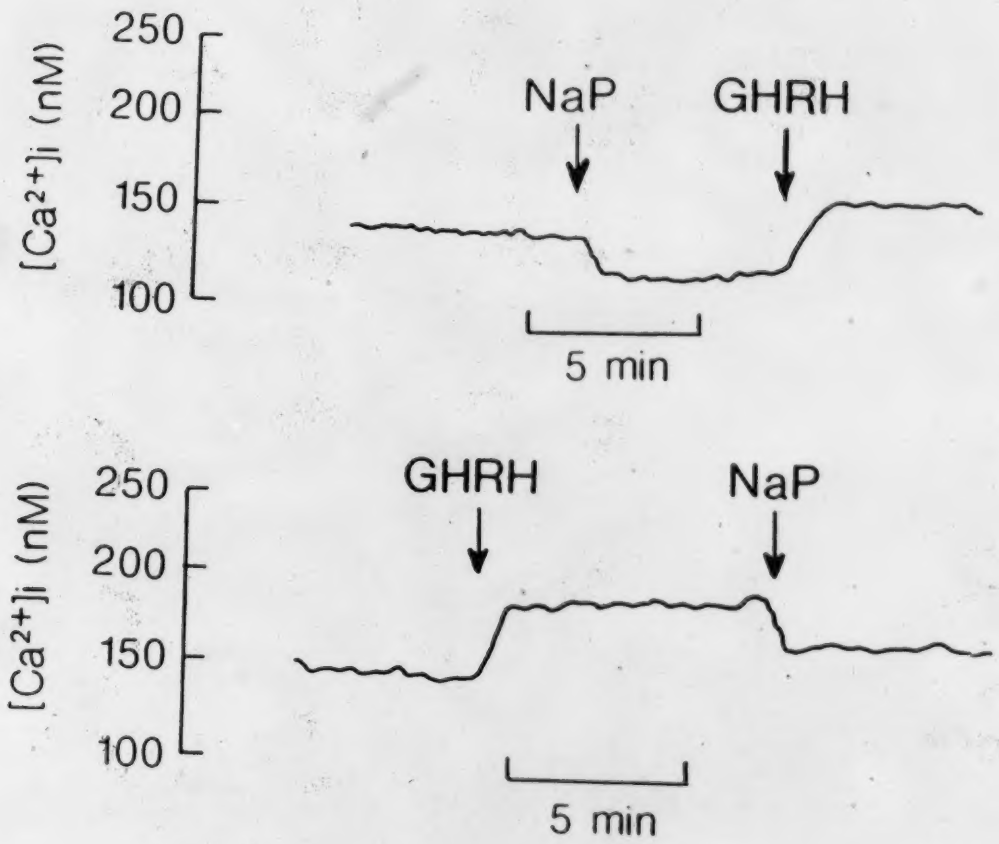

Figure 43. Effects of sodium propionate on $[Ca^{2+}]i$. The effects of sodium propionate (NaP) (30 mM) on a) basal and b) GHRH-stimulated $[Ca^{2+}]i$ was determined. The tracing is representative of three experiments.

The effect on $[Ca^{2+}]i$ by elevation of pHi using ammonium chloride was then studied. Ammonium chloride at a concentration of 30 mM produced a small increase in basal $[Ca^{2+}]i$ levels from 138 $\pm$ 12 nM to 155 $\pm$ 12 nM. Pretreatment with ammonium chloride, however, had no effect on the subsequent GHRH-stimulated increase in $[Ca^{2+}]i$ (Figure 43). The addition of ammonium chloride after treatment with GHRH again resulted in a small increase in $[Ca^{2+}]i$ (Figure 43).

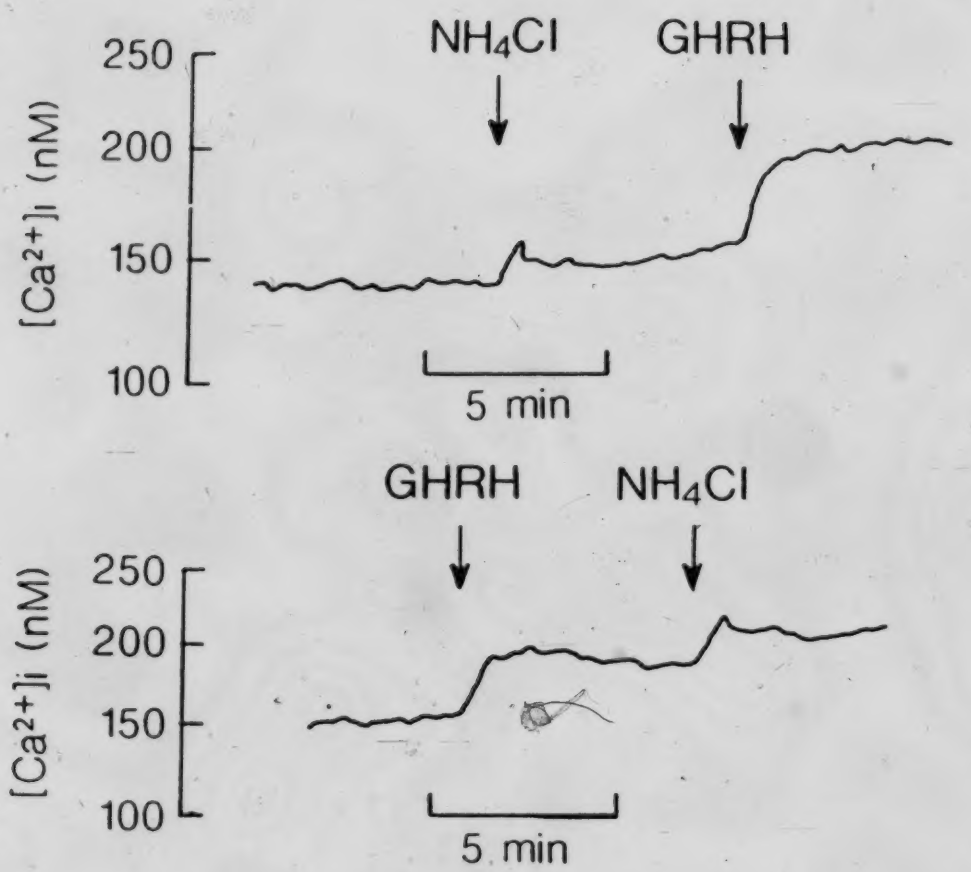

Figure 44. Effects of ammonium chloride on $[Ca^{2+}]i$. a) After stabilisation of the basal $[Ca^{2+}]i$, ammonium chloride (NH_4Cl) (30 mM) was added and the signal monitored; b) To determine the effects on GHRH-stimulated $[Ca^{2+}]i$, GHRH (10 nM) was added followed by ammonium chloride. The tracing is representative of three experiments.

III.6 SUMMARY

TREATMENT	pHi	cAMP released		$[Ca^{2+}]i$		GH secreted	
		Basal	GHRH-stimulated	Basal	GHRH-stimulated	Basal	GHRH-stimulated
Inhibition of Na^+/H^+ antiporter	↓	↔	↓	ND	ND	↔	↓
Elevation of pHo	↑	↔	↑	↑	↑	↑	↑*
Sodium propionate	↓	↔	↓	↓	↓	↔	↓
Ammonium chloride	↑	↔	↑	↑	↑	↔	↓

ND: not determined
* : GH release at 15 min

To summarize, the findings of this study include:

a) GHRH-stimulated cAMP release from the anterior pituitary cells of rat is pH-dependent. A parallel relationship exists between pHi and GHRH-stimulated cAMP release; elevation of pHi leads to elevation of GHRH-stimulated cAMP release and reduction of pHi leads to reduction of GHRH-stimulated cAMP release.

b) Intracellular Ca^{2+} concentration in the anterior pituitary cells also seems to be related to pHi in a parallel manner; elevation of pHi elevates basal and GHRH-stimulated $[Ca^{2+}]i$, reduction of pHi reduces basal and GHRH-stimulated $[Ca^{2+}]i$.

c) Growth Hormone secretion from the anterior pituitary cells is also modulated by changes in pHi; however, the relationship is more complex and depends upon the method used to modulate pHi. These modulatory effects of pHi on GH secretion cannot be explained by the effects of pHi on cAMP release or $[Ca^{2+}]i$ alone.

IV. Discussion

This study was undertaken to extend the knowledge on the regulation and importance of pHi in eukaryotic cells and more specifically, its effects on GH secretion from somatotrophs. Specifically, the modulatory effects of changes in pHi on receptor-mediated intracellular mechanisms involved in GH release from the somatotrophs were determined. Since studies dealing with the regulation of GH secretion have suggested cAMP as an important intracellular mediator, the effect of modulating pHi on GHRH-stimulated cAMP release and subsequently, on GH secretion was measured.

The first step in the study was to evaluate whether the established approaches for modulation of pHi were effective in modulating pHi in the anterior pituitary cells from the rat.

There seems to be general agreement that all animal cells possess a plasma membrane Na^+/H^+ antiporter, which is one of the major regulators of pHi (Roos and Boron, 1981). Therefore, to determine whether somatotrophs possess the antiporter and whether the activity of this antiporter is important in regulating pHi in the anterior pituitary cells, experiments were carried out. To study the activity of the Na^+/H^+ antiporter, amiloride and two of its specific derivatives were used to inhibit it. Since the antiporter maintains pHi by extruding protons in exchange with Na^+, inhibition of this exchange would lead to cytosolic acidification. Indeed, our

direct pHi measurements with BCECF have demonstrated that all three amiloride derivatives reduced pHi by 0.05 units, indicating the presence of an amiloride-sensitive Na^+/H^+ antiporter in the anterior pituitary cells.

Although amiloride and its derivatives have been used extensively as blockers of Na^+/H^+ antiporter, they have other effects, including inhibition of Na^+ and Ca^{2+} channels and of the Na^+/Ca^{2+} antiporter (Kleyman *et al.*, 1988; Garcia *et al.*, 1990). Therefore, to confirm the role of pHi, other approaches to modulate pHi were used.

One of the methods which has been used to modulate pHi is varying the extracellular H^+ concentration. Elevating the extracellular H^+ concentration inhibits the forward Na^+_o/H^+_i exchange leading to cytoplasmic acidification (Aronson *et al.*, 1983). Similarly, lowering the extracellular H^+ concentration activates the Na^+/H^+ antiporter promoting cytosolic alkalinization. Evaluation of effects of changes in pHo on pHi demonstrated that pHi varied linearly with pHo within the limits of 6.6-7.8. It was therefore decided that this would be the second approach used to modulate pHi. Another advantage with this procedure was that since no nonspecific chemicals were used, the results obtained may be more specific.

Another approach employed to acid-load the cells was to use sodium propionate. The advantage of this technique over other methods of inducing intracellular acidification was that it permitted the administration of GHRH in normal sodium

medium. Thus, cellular metabolism and regulatory events were only minimally disturbed compared to other procedures which require slow acidification and changes in ion gradients (Na^+ removal or treatment with nigericin) (Moran *et al.*, 1988). Intracellular pH determinations indicated that sodium propionate reduced pHi by up to 0.15 units.

Several agents have been demonstrated to increase pHi. These include a number of tumor promoters and growth factors. However, these agents have effects other than their stimulatory effect on the Na^+/H^+ antiporter leading to an increase in pHi (Grinstein *et al.*, 1986). Thomas (1984) demonstrated that when cells are exposed to ammonium chloride, the NH_3 rapidly enters and combines with H^+ to form NH_4^+, thereby raising the pHi. Our studies using fluorescent-labelled cells demonstrated that ammonium chloride produced cytoplasmic alkalinization which was maintained for the duration of the study (15 min). Therefore, as in other tissues, ammonium chloride was an effective agent in producing cytoplasmic alkalinization in the anterior pituitary cells.

Having confirmed that these approaches were useful in modulating pHi in the anterior pituitary cells, the next step was to study the effects of these modulatory approaches on cAMP release from the anterior pituitary cells.

The interrelationships between pHi and second messengers have been the subject of investigation in numerous studies. Many researchers have demonstrated that the relationship

between pHi and second messengers (cAMP, Ca^{++}, PKC) are complex varying from tissues to tissues (Grinstein *et al.*, 1987; Huang *et al.*, 1987; Moolenar *et al.*, 1983). In this study it has been shown that reduction of pHi through inhibition of the Na^+/H^+ antiporter, reducing pHo or through sodium propionate significantly reduced GHRH-stimulated cAMP release. In comparison, elevation of pHi through elevation of extracellular pH or with ammonium chloride significantly elevated GHRH-stimulated cAMP release. Therefore, a parallel relationship appeared to exist between pHi and GHRH-stimulated cAMP release in the rat anterior pituitary gland.

The consistency of these results with the different methods employed to modulate pHi clearly demonstrated that GHRH-stimulated cAMP release appears to be a pH-sensitive process. Possible mechanisms with pH sensitivity include the binding of GHRH to its receptor, the coupling between receptor-G-protein-adenylate cyclase, or the pathway through which adenylate cyclase is activated. Consistent with this result, Morand *et al.* (1988) recently demonstrated that the binding of glucagon to its receptor in rat hepatocytes was lower at an acidic pH. They also demonstrated an inhibitory effect of acidosis on the regulatory component of Gs and on the catalytic subunit of adenylate cyclase. Changes in pH sensitivity of adenylate cyclase in rat hepatocytes has also been demonstrated by Johnson (1982). However, in the present study, a direct effect of pHi on adenylate cyclase activity

was unlikely since the basal cAMP release was not affected by changes in pHi. Another possible explanation is an effect of pHi modulation on phosphodiesterase activity since changes in cAMP release and pHi were parallel. However, the absence of an effect of pHi on basal cAMP release made this possibility unlikely.

Having determined the interrelationship between pHi and GHRH-stimulated cAMP release, the next step was to determine the effects of these modulatory approaches on GH secretion. Of the studies done so far, the relationship between pHi and hormone secretion have been variable depending on the tissues studied. Moran *et al.* (1988) demonstrated that parathyroid hormone has an inhibitory effect on the Na^+/H^+ antiporter in cultured opossum kidney cells. In isolated rat hepatocytes, Morand *et al.* (1988) demonstrated that changes in pHo modulate the effects of glucagon.

The role of changes in pHi on the secretion of various pituitary hormones has not been investigated previously. The possible involvement of the Na^+/H^+ antiporter in the secretion of pituitary hormones, however, has been the subject of investigation in two recent studies. The first study demonstrated that in GH_4C_1 cells, activation of TRH receptors leads to a transient rise in $[Ca^{2+}]i$ which in turn leads to activation of the Na^+/H^+ antiporter, and cellular alkalinization. The TRH-mediated effect is Na^+-dependent and can be inhibited by amiloride (Tornquist and Tashjian, 1991).

In the second study, the role of the Na^+/H^+ antiporter on LH release was evaluated using a series of amiloride derivatives. The amiloride derivative with potent inhibitory action on the Na^+/H^+ antiporter had a marked inhibitory effect on the GnRH-stimulated LH release (McArdle *et al.*, 1991).

In the present study, the effect of pHi on the early and late release of GH was characterized. A static culture system was used since the method had the added advantage of comparing the effects of different secretagogues at various concentrations on the same batch of prepared cells.

Unlike the effects on cAMP, the modulatory effects of pHi on GH release 15 min after stimulation appeared to depend on the method through which pHi was modulated. A possible explanation for this variability was the complexity involved in the secretion of GH from the anterior pituitary gland.

Reduction of pHi through the inhibition of the Na^+/H^+ antiporter using amiloride and its two derivatives, reduction of pHo or through sodium propionate significantly inhibited GHRH-stimulated GH release, an effect similar to the effects on GHRH-stimulated cAMP release. This finding suggested that the modulatory effects of pHi through amiloride on GH release appeared to reflect its action on the cAMP pathway.

In contrast, the effects of elevation of pHi on GH release were more complicated. The lack of parallelism between the effects on cAMP and GH release suggested that the effects of pHi on GH release may not entirely reflect its action on

the cAMP pathway. Elevation of pHi by elevating extracellular pH produced a six-fold elevation of basal GH release and a small elevation of GHRH-stimulated GH release. However, elevation of pHi using ammonium chloride had little effect on basal GH release but significantly inhibited GHRH-stimulated GH release. The opposite effects of elevation of pHi by the two different approaches indicated that in addition to the cAMP pathway, other pathways involved in GH secretion may be sensitive to changes in pHi. The possible pathways are Ca^{2+} mobilization (Schettini *et al.*, 1984), increased membrane Na^{+} conductance (Kato *et al.*, 1989a; b), phospholipid hydrolysis (Canonico *et al.*, 1983), activation of PKC (Limor *et al.*, 1989, Cronin *et al.*, 1985; 1986), and activation of the arachidonic acid cascade (Fafeur *et al.*, 1985).

Among the different intracellular mechanisms known, mobilization of Ca^{2+} appeared to be a likely candidate to be affected by changes in pHi since parallel changes in pHi and $[Ca^{2+}]i$ have been reported in several tissues (Grinstein *et al.*, 1985). Also, experiments by a number of investigators have demonstrated that influx of Ca^{2+} was required to express the action of cAMP *i.e.* for the release of GH (Lussier *et al.*, 1991a; b; c). Therefore, an effect of modulation of pHi on the Ca^{2+} pathway would in part explain the different effects on cAMP and GH release. Since the influx of Ca^{2+} has been postulated to be necessary for the release of GH, an effect on this influx by changes in pHi would explain the effects on

basal as well as GHRH-stimulated GH release.

An effect of modulation of pHi on basal and GHRH-stimulated $[Ca^{2+}]i$ release was confirmed by direct determination of $[Ca^{2+}]i$ using fura 2. Compared to other studies, the signals produced by somatotroph-specific secretagogues (GHRH) on $[Ca^{2+}]i$ were lower than those seen with purified somatotrophs since our experiments were performed with mixed cell populations.

It was found that acute elevation of pHo produced a significant elevation of basal $[Ca^{2+}]i$. Since elevation in $[Ca^{2+}]i$ could promote GH release by exocytosis (Lussier *et al.*, 1991), increased basal GH release with high pHi likely occurred through elevation in $[Ca^{2+}]i$. Similarly, acute elevation in pHi using NaOH produced significant elevation of GHRH-stimulated $[Ca^{2+}]i$ explaining the elevation of GHRH-stimulated GH release on elevation of pHo using NaOH. Acute reduction of pHi from 7.2 to 6.6 using HCl failed to produce a significant effect on basal and GHRH-stimulated $[Ca^{2+}]i$. This is probably why reduction of pHo from 7 to 6.6 did not significantly affect basal and GHRH-stimulated GH release.

However, the effects of pHi on $[Ca^{2+}]i$ alone failed to explain the inhibitory effect of ammonium chloride on GHRH-stimulated GH release. Ammonium chloride produced a significant elevation of basal and GHRH-induced rise in $[Ca^{2+}]i$. Therefore, using the above explanation, ammonium chloride should also elevate GH release. However, ammonium

chloride significantly inhibited GHRH-stimulated GH release, suggesting that ammonium chloride possibly modulates one or more of the other intracellular pathways involved in GH secretion. One possible mechanism is change in membrane potential. It has been observed that membrane potential is often somewhat lower after NH_4^+ exposure than before its application (Roos and Boron, 1981). Studies have demonstrated that SRIF produces hyperpolarisation of the cell membrane of the somatotrophs through opening of K^+ channels. This hyperpolarisation inhibits Ca^{2+} influx through the voltage-sensitive calcium channels thus inhibiting GH release (Lussier *et al.*, 1991c). Thus, the inhibition of GH release by ammonium chloride could possibly be through its hyperpolarising effect. Another possibility is that ammonium chloride has an effect on a mechanism beyond elevation of $[Ca^{2+}]i$, for example on the mechanisms involved in exocytosis of the storage granules of GH.

It has been postulated that synthesized GH in the somatotrophs is held in functionally separate pools. Release from these pools occurs in two phases and is postulated to be a function of the granule's position in relation to the cell surface and/or attachment to contractile microtubules (Stachura, 1976). Therefore, to determine the effects of modulation of pHi on the release of the late pool of GH, release of GH was studied 2 h after stimulation.

It was found that inhibition of the Na^+/H^+ antiporter,

reduction of pHo and sodium propionate had inhibitory effects on GHRH-stimulated late GH release. Elevation of pHo elevated basal GH release with no effect on maximum GH release. Ammonium chloride inhibited GHRH-stimulated GH release. In short, all the effects of modulation of pHi on GH release after 2 h closely resembled the effects on GH release after 15 min. It is possible that the effects on the 'early' labile pool of GH overshadowed the effects on the 'late' pool. However, this is unlikely since the GH released after 15 min was removed before the late release was studied. Alternatively, these effects were merely an extrapolation of the effects on the early pool. Another possibility was that the mechanisms or pathways responsible for the release of the late pool of GH were the same as for the release of the early pool. Thus, modulation of pHi would have the same effects on both pools of GH. A limitation of this study was that since a static culture system was used, it may not be possible to separate the individual effects on the two pools of GH.

In conclusion, this study has provided indirect evidence for the presence of an amiloride-sensitive Na^+/H^+ antiporter in the anterior pituitary cells. Modulation of pHi also resulted in a direct effect on GHRH receptor-adenylate cyclase-cAMP pathway. The effects of pHi on GH secretion, however, were more complex indicating that more than one intracellular pathway involved in the secretion of the hormone may be sensitive to changes in pHi. To my knowledge, this is

the first demonstration that pHi plays a modulatory role in the regulation of cAMP content and secretion of GH from the anterior pituitary gland.

This study thus helps extend the knowledge on the regulation and importance of pHi in eukaryotic cells, specifically the somatotrophs. It also provides information on how changes in pHi can modulate receptor-mediated intracellular mechanisms. Establishing a direct link between changes in pHi and different second messengers systems may help explain some of the other physiological consequences of changes in pHi such as cell proliferation and differentiation. It may also help explain some of the clinical effects of acidosis and alkalosis on various tissues.

V. FUTURE STUDIES

a) Effects of pHi on GHRH binding and adenylate cyclase activity

It has been determined that pHi has modulatory effects on GHRH-stimulated cAMP and GH release from rat anterior pituitary cells. The site/sites of action, however, remain to be determined. Since the parallel relationship between pHi and GHRH-stimulated cAMP release indicates an effect on the hormone-receptor-G-protein-adenylate cyclase-cAMP pathway, the exact site of action has to be determined. A study of the effects of pHi on the binding characteristics of the GHRH binding sites and on adenylate cyclase activity would thus be a useful study.

b) Effects of pHi on GH release stimulated by cAMP elevating agents

The studies done so far have also demonstrated that some of the modulatory effects of pHi on GH release are quite different from the effects on the cAMP release. Therefore, a study of the effects of pHi on the post-cAMP events involved in GH secretion would help us understand the effects of pHi better. This could be done by studying the effects of modulation of pHi on GH release stimulated by cAMP elevating agents, for example, forskolin and dibutyryl cAMP.

c) Effects of pHi on GH release stimulated by agents that elevate $[Ca^{2+}]i$

The presence of voltage-sensitive Ca^{2+} channels and their involvement in the basal and GHRH-stimulated GH release have been well established. It has been established that modulation of pHi has varied effects on $[Ca^{2+}]i$. Therefore, to determine whether pHi affects GH release by acting on the Ca^{2+} channels, the effects of pHi modulation on K^+-stimulated GH release would have to be studied.

Since the elevation of $[Ca^{2+}]i$ does not explain the effects of ammonium chloride on GH release, the possibility of effects of pHi on GH release at a site distal to the elevation of $[Ca^{2+}]i$ has to be considered. This can be studied by experiments on the effects of changes on pHi on ionomycin-stimulated GH release.

d) Effects of pHi on other pathways involved in GH release

Apart from the cAMP and Ca^{2+} pathway, other pathways including protein kinase C and arachidonic acid cascade have been postulated to be involved in GH release. A study of the effects of pHi on these pathways may help understand the effects of modulatory effects of pHi. It may also help understand the complex interrelationships between the various pathways involved in the secretion of GH.

e) Interrelationship between pHi and SRIF

Barber *et al.* (1989) have demonstrated that in enteric endocrine cells, SRIF receptors regulate Na^+/H^+ exchange activity. It is therefore important to determine whether SRIF acts via pHi to affect pHi production. Thus the effect of SRIF on pHi has to be determined. To determine if the inhibitory effects of some of the approaches to modulate pHi on GH release are mediated via SRIF, the effect of pHi on SRIF activity has to be characterized.

There is therefore enormous potential for an interesting study along the lines indicated to further our knowledge regarding the functioning of an endocrine organ and the modulatory effects of pHi.

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APPENDIX 1

Inhibitory Potencies of Amiloride Derivatives

NAME	Abbrevia.	Ki (μM) of System Inhibited		
		Na^+ Channel	Na^+/H^+ Antiporter	Na^+/Ca^{2+} Exchange
5-(N-methyl-N-isobutyl) amiloride	MIBA	>300	0.44	135
Dimethyl amiloride	DMA	>400	6.9	550
Amiloride	Amil	0.34	83.4	1100

APPENDIX 2

PHOSPHATE-BUFFERED SALINE

Sodium Chloride	140 mM
Potassium Chloride	2 mM
Sodium Phosphate Dibasic	10 mM
Potassium Phosphate Monobasic	2 mM
pH	7.4

APPENDIX 3

EARL'S BALANCED SALT SOLUTION

Earl's Balanced Salt (Sigma)	8.6 g
Dextrose	30 mM
Sodium Bicarbonate	26 mM
Pen-strep-fungizone	1% (wt/v)
Distilled water	1 L
Osmolarity	310 osm.
pH	7.4

APPENDIX 4

BUFFER FOR GH RIA

Sodium Phosphate Dibasic	10 mM
Sodium Chloride	145 mM
Disodium Ethylenediamine Tetraacetate	25 mM
Bovine Serum Albumin	10 g
Merthiolate	200 mg
pH	7.6

APPENDIX 5

RPMI MEDIUM

RPMI-1640 (Gibco, 10X)	20 mL
HEPES	5 mL
Pen-strep-Fungizone	1%
Distilled water	180 mL
pH	7.2

APPENDIX 6

FURA 2 MEDIUM

Sodium chloride	140 mM
Potassium chloride	5 mM
Calcium chloride	2 mM
Magnesium chloride	1.2 mM
Potassium phosphate monobasic	1.2 mM
HEPES	25 mM
Glucose	6 mM
pH	7.2